FALL 1971

THE UNIVERSITY OF ALBERTA

CHEMICAL INHIBITORS OF NITRATE FORMING PROCESSES IN SOIL

by

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B.Sc.

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES & RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF SOIL SCIENCE

EDMONTON, ALBERTA

FALL 1971

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES & RESEARCH

The undersigned certify that they have read, and
recommend to the Faculty of Graduate Studies & Research
for acceptance, a thesis entitled

CHEMICAL INHIBITORS OF NITRATE FORMING PROCESSES IN SOIL

submitted by James Isao Fujikawa in partial fulfilment of
the requirements for the degree of Master of Science.

Date ..... *October 4ᵗʰ 1971*

ABSTRACT

Seventy chemicals, sulfur and nitrogen-containing organics, organo-nitrogen compounds and polymeric preparations including those of thiourea and formaldehyde were assessed for agricultural potential as inhibitors of nitrate formation in soil. The performance of the organo-nitrogen compounds was generally inferior to that of the nitrogen and sulfur-containing organics. Of the latter group of compounds, those possessing both a nitrogen and sulfur atom on the same carbon atom were particularily effective inhibitors.

118-66-IC, a low molecular weight polymer of thiourea and formaldehyde, demonstrated significant activity; 80% - 85% inhibition of nitrification observed with an application rate of 120 ppm. 118-66-IC proved non-phytotoxic even at concentrations as high as 800 ppm. and, in greenhouse tests, significantly increased grass yields through nitrogen conservation. As a nitrification inhibitor, 118-66-IC remained active in soil for periods exceeding thirteen months in greenhouse tests and thirty months under field conditions. 118-66-IC retarded nitrate formation from urea and ammonium nitrogen sources slightly more effectively than from a nitrite nitrogen source.

ACKNOWLEDGEMENTS

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I. INTRODUCTION

Figures compiled by the Dominion Bureau of Statistics
(Manufacturing and Primary Industries Division) for the
year 1946, show that sales and consumption of mixed ferti-
lizers and fertilizer materials in Canada amounted to
632,000 tons. This figure was doubled in the space of 17
years, fertilizer consumption reaching 1.26 million tons
in 1963. In the last 5 years, a growing synthetic
fertilizer industry together with a demand for increased
productivity has resulted in a continued acceleration
in fertilizer trade.

As an alternative to increased rates and repeated
applications of fertilizers, efforts have been made to
improve the efficiency of present fertilizer products.
Such investigations are especially pertinent to nitrogen
fertilizers, which display largely uncontrolled nutrient
release patterns. The problems associated with such
inefficient patterns of nutrient supply lead to luxury
consumption, hidden hunger, fertilizer toxicity and
nutrient losses. For nitrogen fertilizers nutrient loss
is a significant consequence of ammonification and
nitrification which are biological soil processes leading
to the formation of nitrate nitrogen. The water soluble nit-
rate ion, while tentatively accepted as the nitrogen form

utilized by most plants, is subject to transport and loss through leaching and runoff. Additionally, formation of this ion facilitates volatile nitrogen losses through chemical and biological denitrification.

Several approaches have been taken in attempts to minimize losses and to increase crop recovery of nitrogen added as fertilizer. Apart from management practises, involving split applications and placement, efforts to control the rate of nitrogen release have been made. Degrees of success have been achieved in this respect through development of low or limited solubility nitrogen sources, coated or encapsulated nitrogen sources and chemicals designed to inhibit biological activity such as nitrification and ammonification.

The use of nitrification inhibitors represents a promising, recent approach to conservation of applied fertilizer nitrogen and is a concept worthy of further investigation. To this end, a course of study was undertaken encompassing the following objectives:

1. To establish reliable screening procedures whereby selected compounds may be assessed for their potential as nitrification inhibitors of agricultural importance.

2. From an examination of a wide range of chemical compounds; to characterize and categorize chemical structures and properties associated with the inhibition of nitrification.

3. To isolate and extensively investigate specific nitrification inhibitor(s) warranting possible commercial development. Areas to be considered here are:

    a) Inhibitory activity with respect to applica-rates.

    b) Phytotoxicity with respect to application rates

    c) Product degradation.

    d) Field performance of the chemical.

    e) Product synthesis and manufacturing.

    f) The mechanism of inhibition.

II. LITERATURE REVIEW

1. The Influence of Ammonium Versus Nitrate Nutrition on Plant Growth

Plant roots have the capacity to absorb and utilize a wide variety of nitrogen sources. According to Viets (1965), the uptake of ammonium, nitrate, nitrite, urea and simple organic nitrogen molecules such as amino acids, dipeptides and betaines has been observed in experiments involving plants grown on sand and water cultures. The uptake rates of these nitrogen compounds, although influenced significantly by numerous variables e.g. plant species, environmental factors; differ distinctly. When equally available, plants generally absorb nitrate in preference to ammonium. However, both ions are rapidly assimilated by roots; rates of entry being comparable to ions such as K^+, Rb^+, Cl^- and $H_2PO_4^-$ and faster than those for Ca^{++}, Mg^{++}, and SO_4^{--}. The uptake of urea is faster than that of either ammonium or nitrate (Hirose and Goto, 1961). Generally, in a porous medium like soil, the rate of absorption of nitrogen forms by roots is probably more dependent on the rate of transport to the root surface and on the rate of root extension than on absorption ability of the root (Viets, 1965).

The mechanism of nitrate and ammonium assimilation by plant roots remains largely unelucidated. From comparative uptake studies with K^+, and $H_2PO_4^-$, absorption of nitrate and ammonium by plant roots is postulated to involve, initially, absorption onto a carrier surface (Viets, 1965). The existence of this active transport system for nitrate and ammonium uptake is substantiated in part by the observation that absorption of these ions can occur against concentration or activity gradients two-three hundred times that of the surrounding root solution. Further indications of an active transport system is the fact that the rate of uptake of nitrate and ammonium varies not only with temperature and salt status of the roots but also with O_2 concentration, sugar supply and concentration of energy materials (Broyer, 1951; Hoagland and Broyer, 1936, 1940). In contrast urea assimilation by roots is reported to be a simple diffusion process (Hirose and Goto, 1961).

Assimilated nitrogen forms are combined with cellular carbohydrates in the synthesis of plant amino acids. Curtis and Clark (1950) report a postulated mechanism of plant amino acid synthesis in which carbohydrates are first changed to corresponding fatty acids. Condensation of the fatty acid moiety with ammonium absorbed by the roots is thought to follow. When nitrate is the nitrogen source,

however, synthesis of plant amino acids initially involve an enzyme catalyzed, energy requiring reduction of nitrate to ammonia.

Evidence for nitrate reduction in plants is well documented. The isolation and action of nitrate reductase has been reported by several workers (Hageman and Flesher, 1960; Sanderson and Cocking, 1964; Nightingale, 1948). Additionally, the sequential appearance with time, of nitrite, hydroxylamine, ammonium and organic nitrogen has been repeatedly noted for growing plants supplied with nitrate nitrogen (Curtis and Clark, 1950; Nightingale, 1948).

As the utilization of nitrate nitrogen in plant protein synthesis requires an expenditure of energy over and above that necessary with ammonium nitrogen; logically, plant growth should be favored by the latter nitrogen source. Such a conclusion was drawn by Nightingale (1948). More recently, however, research into this question of ammonium versus nitrate nutrition for plants has revealed a more complex picture.

In a complete cultural solution, plants frequently absorb the nitrogen bearing ions, nitrate and ammonium, more rapidly than other ions present. Substantial shifts in pH can occur with such differential ion absorption

(Curtis and Clark, 1950; Viets, 1965). Should nitrate be the only nitrogen source, plant growth results in a more rapid uptake of nitrate ions. To maintain electrostatic balance, hydrogen ions from the nutrient solution may enter the root cells along with the nitrate ions or bicarbonate ions may be given off by the root cells. The cumulative effect is an increase in solution pH. With available nitrogen totally in the ammonium form, nitrogen uptake by plants results in the rapid uptake of anions as opposed to cations and consequently causes the solution pH to fall. Here, hydroxyl ions may be simultaneously absorbed with the ammonium ions or hydrogen ions may be excreted by the root cells. In soil, the previously described pH effects are accentuated by pH changes concurrent with nitrification and are minimized by the buffering capacity of the soil.

Under conditions of poor soil aeration, nitrate is reported to be superior over ammonium as a nitrogen source for plant growth (Arnon, 1937; Viets, 1965). In such cases, nitrate is thought to function as a terminal electron acceptor or "sink" for the root cells of these higher plants. Further, under such conditions of poor soil aeration, the addition, in non-toxic quantities of manganese and copper ions to the culture solution increased the growth of ammonium fed plants but had little effect on

nitrate fed ones. The beneficial effect imparted by these metal ions is thought to be due to their catalytic function in the promotion of redox processes in the plant.

Whether a plant absorbs its nitrogen in the cationic (NH_4^+) or anionic (NO_3^-) form profoundly influences the uptake of other essential nutrient ions. The uptake of nitrate adversely affects the absorption of chloride, sulfate and phosphate ions (Rehm, 1969; Viets, 1965; Nightingale, 1948). Nitrate and K^+, however, mutually enhance each other with regard to uptake (Nightingale, 1948). Ammonium absorption depresses the uptake of mineral cations, particularily K^+ and results in a lower organic acid content in the plant as the absorbed ammonium is rapidly combined into amino acids and amides (Viets, 1965).

The organic acid content of plants was related by deWit (1963) to growth response. The maximum growth of plants was associated with a constant value for the expression (C-A) per unit dry weight. (C-A) represented the total organic acid content; C, the sum in equivalents of cations (K^+, Na^+, Ca^+, Mg^+) and A, the sum in equivalents of inorganic anions (NO_3^-, $H_2PO_4^-$, SO_4^-, Cl^-). As other cations compete unfavorably with ammonium uptake, the resulting (C-A) stress was thought to be responsible for the lower growth rate noted with ammonium fertilization

(Viets, 1965).

Exhaustive greenhouse and field growth trials have been conducted comparing the relative merits of ammonium versus nitrate nitrogen sources. If plant yields did differ, the exact reason for this difference was seldom established because of the great number of possible explanations. Yield differences may be attributed, in part, to established differences in the efficiency of the two nitrogen sources. Thus under greenhouse conditions, where leaching and the possibility of denitrification are minimized by careful watering, nitrate uptake tends to be superior to that for ammonium (Broadbent and Nakashima, 1968; Atanasih, West-phal and Banerjee, 1968). Under field conditions, however, ammonium uptake surpasses that of nitrate (Nelson, 1967). Here, NH_4^+, through absorption onto clay and other colloidal soil material, remains positionally more available than nitrate. Aside from the obvious, however, ammonium and nitrate applications impart shifts in soil pH which influence other mineral constituents of the soil and may create complex side effects affecting plant growth (Viets, 1965). Thus yield increases of Milo and Ladino clover under ammonium fertilization were traced to increased uptake of both indigenous and applied zinc; a consequence of decreased soil pH (Viets, 1957). Similarily, better crop yields obtained with ammonium as opposed to nitrate

fertilization were attributed to the influence of ammonium carriers in lowering soil pH, thus increasing the soluble PO_4^- in the soil and enhancing uptake by crops (Lorenz and Johnson, 1953).

2. Aspects of Nitrification in Soil

Nitrification has remained a topic of active interest and attention since its initial discovery (Pasteur, 1862; Schloesing and Muntz, 1877) and characterization (Warington, 1878, 1879; Winogradsky, 1890, 1891, 1899). Consequently, the fundamental aspects of this biological process are now well defined and generally accepted with little controversy.

A. Influencing Environmental Factors

Nitrification may be defined as a biological process, naturally occurring in varied environments ranging from soil to activated sewage, in which ammonium is oxidized in a stepwise manner to nitrate. The rate and extent of this transformation has been linked to a number of definite environmental factors (Meikeljohn, 1954; Alexander, 1967). O_2 is an obligate requirement for nitrification. Light, however, adversely influences this process. Temperature markedly affects the transformation rate of ammonium to nitrate. 30-36°C is generally accepted as the optimum

temperature range; however, nitrification does proceed,
although at a very slow rate, at temperatures below 5°C
and above 40°C. Moisture status is a factor governing
nitrification in soil. The optimum moisture level varies
considerably with different soils, but nitrate generally
appears most rapidly at one-half to two-thirds the moisture
holding capacity. The autotrophic nitrifying bacteria are,
however, highly resistant to drying in soil (Meikeljohn,
1954). pH bears a highly significant correlation to nitrate
production. The rate of nitrification falls off markedly
below pH 6.0 and becomes negligible below pH5.0. Above
pH 9.4, excess alkalinity depresses nitrifying activity.
The optimum hydrogen ion concentration for nitrification
has been placed on the alkaline side of neutrality; suggested
pH values ranging from 7.8 (Alexander, 1967) to 8.8 (Meikel-
john, 1954). Since the nitrifying organisms involve a
number of species, and since these organisms display an
ability to adapt to moderately acidic and alkaline environ-
ments, differences in the observed optimum pH value for
nitrification are expected.

B. Microbiology of Nitrification

Although considerable reference has been made to
heterotrophic nitrification (Elylar and Schmidt, 1959;
Marshall, 1962; Jensen, 1951; Alexander, 1965, 1967),

nitrification has been chiefly attributed to members of the
Nitrobacteriaceae, a family of autotrophic bacteria
classified under the order Pseudomonadales. Members of this
family display considerable variety in morphology (Alexander,
1967), varying in shape to include rods, ellipsoids, cocci
and spirilla. Some species may possess polar flagella,
others are characterized by the formation of zooglea. All
members of the family, however, lack endospores.

Of the seven genera recognized in the family Nitrobac-
teriaceae, Nitrosomonas and Nitrobacter are considered the
major nitrifying chemoautotrophs. Members of the genus
Nitrosomonas characteristically oxidize ammonium to
nitrite, lack zooglea and feature flagellated and non-
flagellated ellipsoid cells 1.5 microns by 1.0 microns in
dimension (Meikeljohn, 1954). N. europaea and N. monocella
are two species commonly associated with this genus.
Members of the genus Nitrobacter also lack zooglea and
exhibit flagellated and non-flagellated ovoid cells;
dimensions being 1.0 microns by 0.8 microns. These auto-
trophs, however, oxidize nitrite to nitrate. N. agilis
and N. winogradsky constitute two prominent Nitrobacter
species.

The physiology of the nitrifying bacteria, specifically
Nitrosomonas and Nitrobacter, has been closely linked

with certain features revealed in recent ultrastructure studies (Murray and Watson, 1965). Electron micrographs of Nitrosomonas show that the cytoplasm contains intrusive, paired lamellae which may or may not remain connected to the periphery. These structures do not fuse nor do they form regular associations. Similarily Nitrobacter exhibits intrusions of the plasma membrane. The intra-cytoplasmic membranes, here, are close and regularily spaced. They are arranged over and shape the poles of the cell. Further, the intrusive lamellae of Nitrobacter displays a very dense outward facing layer. It is proposed that this region of extreme density might represent a zone of concentration of some metal-enzyme complex such as cytochromes or copper enzymes. These findings appear in good accord with the belief that energy processes carried out by these chemautotrophs are membrane linked.

C. Biochemistry of Nitrification

The intermediary metabolism of Nitrosomonas spp. has not been fully elucidated. A number of facts regarding this subject, however, have been established and documented.

1. Chemautotrophs of the genus Nitrosomonas derive energy for growth solely from the oxidation of ammonium to nitrite (Alexander, 1965, 1967; Meikeljohn, 1954).

2. In the biological transformation of ammonium, the

latter is first converted to hydroxylamine and then to nitrite, presumably via an unidentified and unstable intermediate at the nitrogen oxidation level (+1) represented by (:NOH). The latter species may further dissociate t$_0$ N$_2$O and N$_2$ under anaerobic conditions (Aleem, 1962; Alexander, 1965; Kluyver and Donker, 1926; Lees, 1955). The overall oxidation process may be prepresented as follows:

$$NH_4 + 1.5O_2 \longrightarrow 2H^+ + NO_2- + 66.5 \text{ kcal.}$$
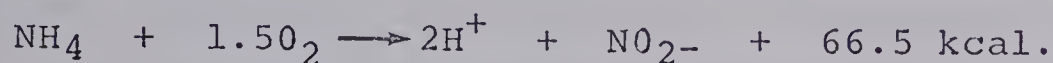
Stoichiometric verification has been achieved by O$_2$ uptake studies.

3. For <u>Nitrosomonas</u> the free energy efficiency ranges from 5-14% and the biochemical efficiency i.e. ratio of inorganic nitrogen oxidized to CO$_2$ carbon assimilated varies from (35-70):1 (Alexander, 1967).

4. Ammonium oxidation at the cell-free level has not been satisfactorily achieved. Anderson (1959) has postulated that cell-free <u>Nitrosomonas</u> extracts failed to oxidize ammonium because oxidation to hydroxylamine is an endergonic reaction ($\Delta F = 4.7$ kcal.) requiring energy rich PO$_4$ for activation. Cell-free extract preparation conceivably disrupts the energy coupling mechanisms. The conversion of hydroxylamine to nitrite, however, has been achieved by cell-free extracts of <u>N. europaea</u> when supplied by electron acceptors such as mammalian cytochrome c or phenazine methosulphate (Nicholas and Jones, 1960).

Further, a forty-fold purification of the hydroxylamine

oxidase was achieved.

5.   The biological oxidation of hydroxylamine has been

definitely linked to a cytochrome system; a_1- and a-like

components being detected in the cytochrome oxidase (Aleem

and Lees, 1963). Campbell and Aleem (1965) have suggested

that it is reasonable to expect either or both such cyto-

chrome components to be operative in ammonium oxidation.

6.   In crude cell-free extracts of Nitrosomonas,

oxidation of hydroxylamine and the concomitant esterification

of inorganic phosphate has been noted (Delwiche, 1961).

Details concerning the energy yielding processes of

Nitrobacter spp., although incomplete, have been more fully

characterized than those of Nitrosomonas. A summary of the

pertinent findings is presented below:

1.   Nitrobacter spp. are chemautotrophs deriving their

energy for growth from the oxidation of nitrite to nitrate

(Alexander, 1965, 1967; Meikeljohn, 1954; Aleem, 1962).

2.   Stoichiometrically, the biological oxidation of

nitrite obeys the following chemical equation:

$$NO_2^- + 1/2O_2 \longrightarrow NO_3- + 17.5 \text{ kcal.}$$

3.   The Nitrobacter spp. have a free energy efficiency

of 5-10% and a biochemical efficiency i.e. N:C of (76-135):1

(Alexander, 1967).

4. Active cell-free nitrite oxidizing systems have
been obtained by sonic disintegration of Nitrobacter
cells. The nitrite oxidizing activity has been found to
reside solely in the red particulate fraction (Alexander,
1958).

5. The nitrite oxidizing system of Nitrobacter spp.
initially involves a nitrite-cytochrome c reductase which
mediates the oxidation of nitrite and reduction of
cytochrome c. The further transfer of electrons from
cytochrome c (λMax. = 520, 551 millimicrons) to a_1 (λ Max.
= 585,438 millimicrons) and finally to O_2 is catalysed by
a cytochrome oxidase (Aleem and Nason, 1959, 1963; Lees
and Simpson, 1957; Nason, 1962). These oxidation and
reduction processes taking place in the electron transport
chain are coupled with inorganic phosphate esterification
i.e. ATP formation (Aleem and Nason, 1960; Nason, 1962).

Evidence exists suggesting that the intermediary
metabolism of Nitrosomonas and Nitrobacter is intimately
linked to several metallic cofactors. In incubation
tests assessing nitrite formation, Lees (1948) noted
a definite stimulatory effect with the addition of
ferrous ion in microgram quantities. Trace additions in
microgram amounts of ferrous plus cuprous ions and ferrous
plus cuprous plus Zn^{+2} respectively, proved more stimula-
tory than ferrous ion alone. A trace element solution

(Li,B,Al,Sn,Mn,Ni,Co,Ti,I,Br) together with ferrous,
cuprous and Zn^{+2} further enhanced nitrification suggesting
that a proper balance in quantity as well as species of
metallic elements is important. Significance is attached
to the observation that inhibition of nitrification,
prompted by the addition to soil of compounds known to
strongly chelate cuprous and Zn^{+2} was reversed by the
introduction of supplementary quantities of Cu^{+2}, and to
a limited extent, Fe^{+2}. (Lees, 1946, 1948). Further, in
cell-free studies, Aleem and Alexander (1958) have
observed that either Fe^{+2} or Fe^{+3} stimulate nitrite oxida-
tion.

3. The Consequences of Nitrification with Respect to the
    Conservation of Soil Nitrogen

In terms of the conservation of soil nitrogen and the
efficiency of applied fertilizer nitrogen, the consequences
of nitrification often prove undesirable. Recoveries
in harvested crops of nitrogen that has been released from
the soil or added as fertilizer are repeatedly less than
50% (Allison, 1955). The bulk of such nitrogen losses has
been linked to the probable fates of soil nitrate nitrogen.

A. <u>Leaching Losses</u>

As the end product of nitrification, nitrate constitutes the dominant soil nitrogen form (Ray, McGregor and Schmidt, 1957; Alexander, 1965; Viets, 1965). Due to its extreme water solubility and its anionic character which inhibits adsorption onto soil colloids, nitrate is readily transported by saturated and unsaturated movement of water. The removal, through leaching, of nitrate from the rooting zone of plants constitutes a major mechanism of inorganic nitrogen loss.

That nitrate is more susceptible to leaching than ammonium has been amply verified. The following constitute illustrative examples:

1. Wagner (1958) conducted a comparative study of nitrate and ammonium in fine sandy loam under irrigation. With 600 lb./acre of broadcasted and banded nitrogen and 6.8 inches of water moving through the profile, nitrate was found to extend in significant concentrations throughout the 22.5 inch profile. Ammonium did not move more than 3 or 4 inches under identical conditions.

2. Following laboratory leaching trials employing various nitrogen forms, Akhundov (1967) reported that after three leachings; these being after 7, 22 and 70 days,

the cumulative losses for amide nitrogen were only 3-7%
while that for nitrate nitrogen under identical conditions
was 84%.

Leaching losses of soil nitrate have generally been
measured in lysimeter experiments or in controlled field
drainage plots by measuring the nitrogen content in the
effluent (Chapman, 1949; Bizzell, 1944; Allison, 1955;
Owens, 1960). While the validity of quantitative conclu-
sions drawn from such studies suffer from obvious limita-
tions in experimental technique (Allison, 1955), such
investigations have qualitatively defined a number of
factors influencing the amount of nitrogen lost through
leaching. The more important of these variables are:

(a) Form and amount of soluble and unabsorbed
nitrogen present or added (Nelson, 1953; Allison, 1955)

(b) Time and amount of rainfall (Wetselaar, 1962)

(c) Infiltration and percolation rates as influenced
by soil composition, soil texture, depth of soil profile
and surface treatment of soil (Bates and Tisdale, 1957;
Wetselaar, 1962).

(d) Moisture status and water-holding capacity of
the soil.

(e) Presence or absence, nature and growth character-
istics of cover crop (Allison, 1955).

(f) Rate of evapotranspiration (Allison, 1965).

(g) Rate of nitrogen removal by vegetation (Allison, 1965).

(h) Rate and extent of capillary rise initiated by surface evaporation i.e. rate of recharge of surface soil horizons by subsurface salts (Gardner, 1958).

(i) Height of the existing water table (Kolenbrander, 1961; Owens, 1960).

The significance, agriculturally, of inorganic nitrogen leaching losses is variable. In arid and semiarid regions, removal of soluble nitrogen forms from the rooting zone of plants is not expected to occur unless irrigation practises are followed. In areas of higher rainfall, leaching losses warrant increasing consideration. Allison (1955), in evaluating an extensive review of nitrogen balance studies conducted throughout the United States, expressed the opinion that a "large proportion" of the 25-60% nitrogen loss could be attributed to leaching.

B. Denitrification Losses

Unaccounted nitrogen deficiencies, averaging 15% in magnitude, have been repeatedly reported in nitrogen balance studies (Allison, 1955; Broadbent, 1965). Such deficits have been attributed to volatile nitrogen losses;

the resultant of biological and chemical reductive
transformations of nitrate nitrogen in soil.

Conventionally, the biological reduction of nitrate
and nitrite to volatile gases such as nitrous oxide and
molecular nitrogen is specifically defined as the process
of denitrification. In a broader sense, however, the
latter term may also denote chemical mechanisms of gaseous
nitrogen loss; exclusive of ammonia volatilization.

Occurring under aerobic conditions, chemo-denitrifi-
cation encompasses the reactions of nitrous acid or
nitrites in soil. Various pathways of chemical denitrifi-
cation have been proposed and investigated (Gerretson and
de Hoop, 1957; Allison, 1955, 1963; Tyler and Broadbent,
1960; Clark, 1962). Four mechanisms of chemo-denitrification
are commonly recognized and cited (Broadbent, 1965; Alex-
ander, 1967).

1. The chemical decomposition of HNO_2 at low pH
values. Below pH 5.5, nitrous acid, a product of the
nitrite ion in water, decomposes to yield nitric oxide
according to the reaction:

$$3HNO_2 \longrightarrow NO \ + \ H_2O \ + \ HNO_3$$

Several possible fates are suggested for the NO formed.
Being volitile, NO may escape and be lost to the atmosphere.
Further oxidation of NO may occur to yield nitrogen

dioxide; also a volatile i.e.

$$2NO \longrightarrow O_2 + 2NO_2$$
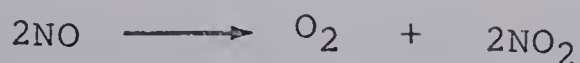
The reaction of NO_2 with water is possible and yields HNO_3 and NO or HNO_2. The regenerated NO and HNO_2 may again participate in the reactions outlined previously.

In neutral or alkaline soil little, if any, NO formation is anticipated. In aerated acid soils, the immediate oxidation and hydration of NO to HNO_3 is expected. As such, large losses of nitrogen from soils as NO is thought unlikely (Gerretson and de Hoop, 1957; Broadbent, 1965).

2. The reaction of HNO_2 with alpha-amino acids. Below pH 5.0 and under conditions of O_2 stress, alpha-amino acids, in the presence of HNO_2, may undergo the Van Slyke reaction to yield molecular nitrogen.

$$RCH(NH_2)COOH + HNO_2 \longrightarrow RCH(OH)COOH + H_2O + N_2$$

The Van Slyke reaction is considered to be an insignificant pathway of volatile nitrogen loss in view of the required reaction conditions. Such conditions are known to inhibit nitrification and bio-denitrification; primary processes of nitrite and hence HNO_2 formation (Allison, 1952; Jones, 1951; Broadbent, 1965).

3. The reaction of HNO_2 with ammonia and urea. Under reaction conditions characteristic of the Van Slyke reaction,

compounds such as ammonia, urea, methylamine, pyrimidines
and purines spontaneously yield molecular nitrogen in
the presence of HNO_2.

e.g. NH_3 + $HNO_2 \longrightarrow N_2$ + $2H_2O$

The rate of N_2 release varies with the reactant and is
most rapid with ammonia.

Assessed from a quantitative standpoint, this mechanism
of volatile nitrogen loss is considered insignificant.

4. The reaction of NO_2- with soil constituents. The
accelerated chemical loss of nitrogen from nitrite in soil
has been observed and linked to soil organic matter
constituents, possibly organic-reducing compounds conceivably
of microbial origin (Broadbent, 1965). Experimental
verification of this theory is, however, limited.

The major mechanism of volatile soil nitrogen loss is
microbiological denitrification (Alexander, 1967). The
bio-reduction of nitrate to nitrogen volitiles, first
reported by Gayon and Dapeit (1886), is a process now
largely characterized as to biochemistry, microbiology and
influencing environmental factors.

Microbiological or enzymatic denitrification is a
transformation accomplished by facultative anaerobes
capable of using nitrate as the terminal electron or

or hydrogen acceptor under conditions of oxygen stress

(Bremner, 1958 a; Alexander 1967). Under aerobic conditions,

the reducing power created by enzymatic dehydrogenations

of organic or inorganic substrate is linked to oxygen with

the subsequent formation of water. In heterotrophic or

autotrophic nitrate respirations, the reducing power is

coupled with oxidized states of nitrogen, specifically

nitrate, nitrite and nitrous oxide.

While the biochemical basis for microbial denitrifi-

cation has been established, the exact mechanism of this

process remains obscure. A summary of current knowledge,

suggests the following pathway:

(Alexander, 1967;
Bremner, 1958a)
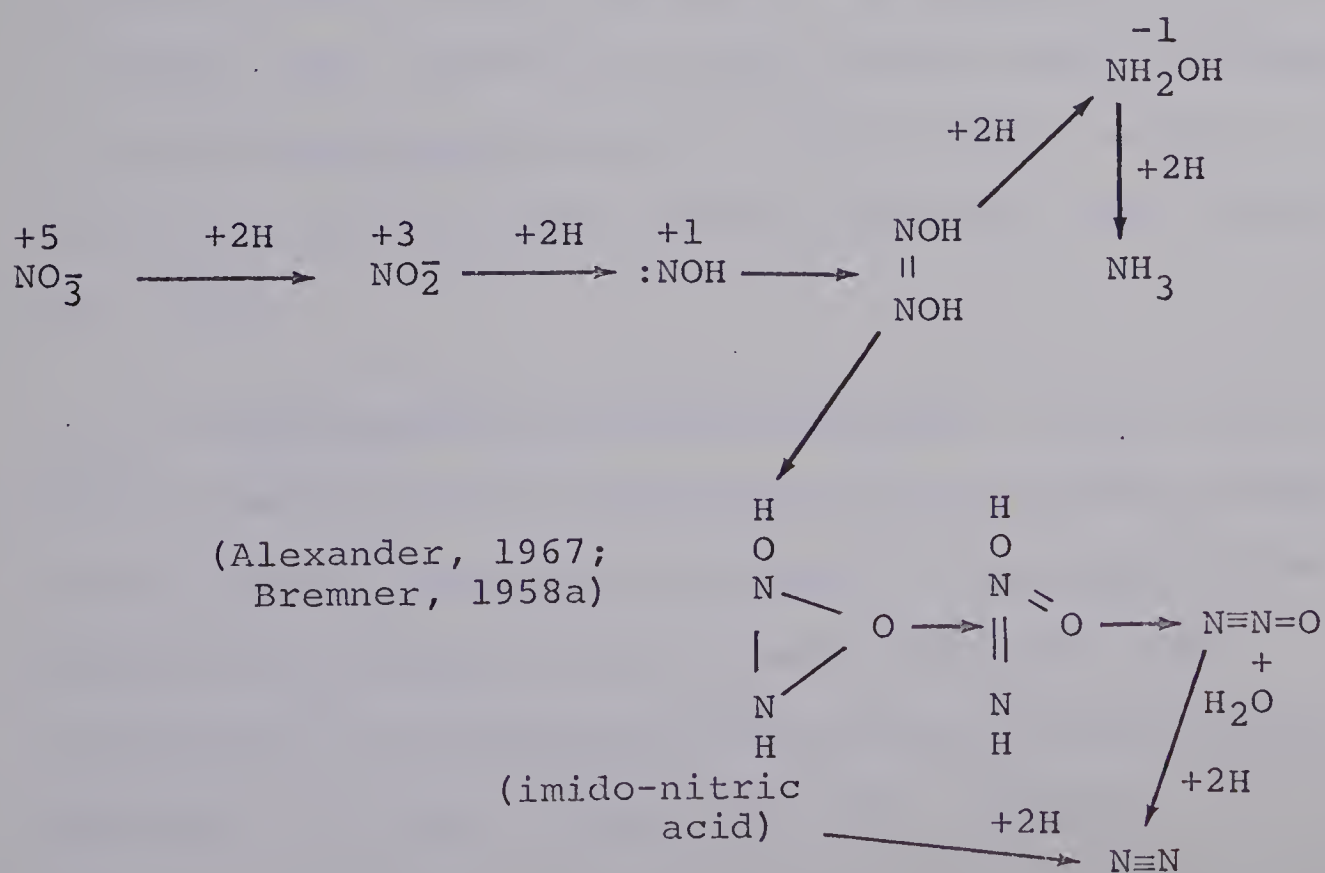
The pathway shown assumes that the reduction of nitrate to ammonia proceeds by a sequence of two electron changes. The mediating enzymes; nitrate, nitrite, hyponitrite and hydroxylamine reductases, have been partially characterized (Nason, 1962). Mo is required for nitrate reductase. Mn is a cofactor for the hydroxylamine enzyme while Cu and Fe are functional in nitrite and hyponitrite reductases (Alexander, 1967).

The primary products of this pathway, namely NO_3^-, NO_2^-, N_2O and N_2 have been repeatedly identified in laboratory and field studies involving N^{15} labelling and gas chromatography techniques (Cooper and Smith, 1963; Cady and Bartholomew, 1960). In addition to the nitrogen gases N_2O and N_2, NO is often detected, especially under conditions of low pH. The formation of this gas has been attributed to the chemical dismutation of nitrite under low pH conditions, yielding HNO_3 and NO (Alexander, 1967; Broadbent, 1965).

(a) Microbiology of Denitrification: Since transformations such as ammonification constitute energy-yielding processes for the microorganisms concerned in denitrification, growth of the latter is not strictly dependent upon the reduction of nitrate and the potential denitrifying population is large; counts of five million

per gram quoted for soil sampled from arable fields (Alexander, 1967).

The capacity for true denitrification is limited to surprisingly few bacterial genera. Fungi and actinomycetes are not implicated. The active species, virtually all facultative anaerobes, are members of the genera Pseudomonas, Achromobacter, Bacillus and Micrococcus.

Several chemautotrophs are capable of reducing nitrate to N_2. Micrococcus denitrificans, a facultative autotroph, develops in air or anaerobically with either organic compounds or H_2 as a source of energy and O_2 or NO_3^- as the terminal electron acceptor. Thiobacillus denitrificans, a sulfur oxidizing chemautotroph, differs from other Thiobacillus spp. by its ability to proliferate anaerobically providing nitrate is available.

(b) Environmental Conditions Influencing Denitrification: The magnitude and rate of denitrification is markedly affected by the environment. Chief among the environmental influences are the nature and amount of organic matter, aeration, the moisture status, soil acidity, and temperature (Alexander, 1967).

In water-logged soils, nitrogen volatilization is markedly enhanced by the addition of carbonaceous materials. Effectiveness of organic nutrients in promoting denitrification is proportional to their availability i.e. a greater stimulatory effect is noted with readily decomposable compounds such as simple sugars or organic acids than with less fermentable straws or grasses.

Necessarily O_2 availability is directly related to denitrification. Aeration affects the transformation in two contrasting ways. Denitrification proceeds only when the O_2 supply is insufficient to satisfy the immediate microbiological demand but at the same time O_2 is necessary for the formation of nitrite and nitrate which are essential for denitrification. Thus, for gaseous losses in fields not receiving nitrates, there must be either aerobic microenvironments for the nitrifying bacteria and anaerobic sites for the denitrifiers or alternations of periods of good and bad aeration e.g. paddy soils.

In well-drained soils, nitrogen volatilization is related to the moisture content. No losses occur at moisture levels below 60% of the water holding capacity of the soil regardless of carbohydrate supply, nitrate con-

centration or pH. Above this figure the rate and magnitude of denitrification is correlated directly with the moisture regime.

Denitrifying bacteria are sensitive to high hydrogen ion concentrations and hence the denitrifying population does not become large enough to account for economic losses until pH 5.5 or greater. The reported optimum pH is 8.0 - 8.6 (Alexander, 1967; Bremner, 1958b).

Denitrification is markedly affected by temperature. The rate of transformation increases rapidly with rise in temperature from 2 to 25°C. The optimum temperature for the reaction is at 25°C and above. The transformation is still rapid at elevated temperatures and will proceed at 60° to 65°C but not at 70°C (Bremner, 1958b).

4. The Control of Plant Nitrogen Supply

Nitrogen fertilizer forms, presently in common use, display largely uncontrolled rates of nutrient release. Such patterns of nitrogen supply prove highly inefficient; failing to satisfy the changing nitrogen requirements of the plant and leading to significant nutrient loss through leaching and denitrification. Although a number of corrective approaches to this problem have been proposed, few have been implemented (Parr, 1964, 1967).

The control of nitrogen supply has been achieved with varying success by the use of limited solubility nitrogen sources. Compounds such as urea-formaldehyde polymers, coal-based fertilizers, oxamides, glycourils and hexamines constitute illustrative examples.

Urea-formaldehyde polymers consist of a continuous series of low molecular weight polymers, the methyleneureas, in which urea molecules are linked together by methylene groups from formaldehyde (Hays, 1967). Applied ureaforms are a mixture of three separable fractions (Hays, 1966, 1967; Kaempffe and Lunt, 1967; Nommik, 1967). The first is that soluble in cold water and incorporates those ureaform molecules generally containing 2-3 urea moieties per molecule. This fraction is nitrified in the space of 1-2 weeks. The hot water soluble - cold water insoluble fraction exhibits 4-5 urea groupings per ureaform -molecule and is nitrified in 1-2 months. That fraction containing 7-8 urea moieties per molecule requires several years to nitrify and is insoluble in hot water. While the relative proportion of these three fractions largely determine the performance of a given ureaform, environmental factors such as pH, moisture, and temperature greatly influence the slow release properties of this product.

A recently introduced slow nitrogen release product is ammonium oxidized nitrogen enriched coal, developed by the Research Council of Alberta. In the syntheses of the latter, powdered sub-bituminous coal, previously oxidized with HNO_3 is reacted with O_2 and NH_3 at approximately 570°C. Most of the nitrogen contained in this coal derived fertilizer (20.1%) occurs in heterocyclic forms and is thus not readily available (Beaton, 1967).

Capsulated or coated water soluble fertilizer nitrogen forms represent an alternate approach to the control of nitrogen supply. Essentially these products consist of incapsulated water soluble, granular fertilizers. The hydrophobic capsule membrane is of variable composition; resins, plastics, waxes, paraffins, asphaltic compounds and elemental sulfur all reported possibilities (Lunt, 1967; Parr, 1967; Powell, 1968; Rindt, 1968). Coatings constitute approximately 15-12% of the total weight of the final product, hence nitrogen sources chosen for capsultation must contain a high proportion of the desired nutrient e.g. NH_4NO_3.

The rate of nutrient release is governed by the characteristics of the capsule coverings (Brown, 1966; Lunt, 1962; Oertli, 1962). The latter vary in thickness

but are generally one of three types; semi-permeable,
perforated or solid-impermeable (Brown, 1966; Parr, 1967).
These products have been successfully adapted for use
in the multimillion dollar nursery industry of Southern
California as well as for specialized crops such as
tobacco and turfgrass. Here the value of the crop offsets
the high initial costs of fertilization.

The development of chemicals to repress or inhibit
the proliferation of soil microbial groups or the enzymes
specifically responsible for the biochemical oxidative
transformations of nitrogen offers another means of
increasing fertilizer efficiency and promoting soil
nitrogen conservation. Urease and nitrification inhibitors
such as acetohydroxamic acid (Jones, 1968) and N-Serve
(Goring, 1962a, 1962b), respectively, constitute illustra-
tive examples.

5. <u>Nitrification Inhibitors</u>

As chemautotrophs, <u>Nitrosomonas</u> and <u>Nitrobacter</u>
elaborate all their cellular material from CO_2 and inorganic
nutrients (Alexander, 1967); energy requirements for this
reductive assimilation of CO_2 (120 kcal. per carbon atom)
being generated by the primary oxidation of ammonium or
nitrite to nitrite or nitrate respectively. Therefore, in

the case of nitrification by the chemautotrophs, two distinct mechanisms of inhibition are possible (Lees, 1963).

Inhibition of nitrification may involve the primary oxidation reactions or its associated system of energy generation. Characteristically, in this case, the oxidation of ammonium or nitrite will cease regardless of whether or not a large population of appropriate organisms is present in the system. Alternately, the reductive assimilation of CO_2, which may involve a system similar to the Calvin cycle and which must involve a multi-staged and complex energy coupling system linking the processes of primary oxidation and reductive CO_2 assimilation, may be inhibited. Here the oxidation of ammonium and nitrite will continue in a system enriched in, or saturated with the appropriate organisms, but will virtually stop in a small or proliferating population of autotrophic nitrifiers.

A considerable number of organic and inorganic compounds, noted to inhibit nitrification have been documented in the literature (Warington, 1878, 1879, 1884, 1891; Monroe, 1886; Winogradsky, 1891, 1899; Meyer-hof, 1916; Lees and Quastel, 1945, 1946; Quastel and Scholefield, 1951; Tomlinson, 1966). Generally such

compounds appear to fall within the categories of general
cell poisons, nitrogen and/or sulfur containing organics,
lipoid soluble substances or electrolytes, particularily
heavy metals (Meiklejohn, 1954). General inhibitors
of nitrification have been reported as well as those
specific in action against the Nitrosomonas spp. or the
Nitrobacter spp.

A. General Inhibitors of Nitrification

(a) Simple Organics: Originally proposed by Winogradsky
and Omeliansky (1899), organic matter has long been
considered inhibitory to the chemautotrophic nitrifying
population. Considering their ecology in soil, severe
doubt exists as to the validity of these original findings.
More recently, it is thought that the depressed nitrifying
activity associated with the introduction of organic matter
to soil is due largely to the stimulation of heterotrophic
populations and the consequent increased assimilation of
available nitrogen (Quastel and Scholefield, 1951).
Whether various organics stimulate, inhibit or leave un-
affected the nitrifying organisms is a question which has
commanded considerable past research attention.

Components of laboratory growth media have been
reported as inhibitory to nitrification (Lees, 1963).

Inhibition with peptone has been tentatively attributed to the presence of free amino acids and the subsequent chelation of essential trace metals. The induction of wasteful carboxylations has been suggested as a possible cause for inhibition with decimolar quantities of organic acids such as formate, acetate and butyrate. In the case of glucose toxicity, mannose, present as a contaminant or through chemical change from glucose, has been suggested as the responsible agent.

(b) Nitrogenous Organics: Organo-N compounds, notably certain aliphatic and aromatic amincs, alkaloids, pyridines, guanidines, alkyl carbamates (urethanes), and quinones prove highly inhibitory to nitrification (Quastel and Scholefield, 1951; Meikeljohn, 1954; Tomlinson, 1962). Significant aspects concerning these compounds follow.

(i) Aliphatic and Aromatic Amines: Effective at concentrations ranging from $10^{-2} - 10^{-4}$ M. (McBeath, 1962), these compounds inhibit nitrification non-specifically. The toxicity of aliphatic amines generally surpasses that of the aromatics; however, inhibitory activity of the amines per se increases with increasing lipoid solubility and pH (Meikeljohn, 1954; McBeath, 1962). Of the aromatic amines, aniline and its substitution products prove most toxic (Meikeljohn, 1954; Hoflich, 1968).

(ii) <u>Alkaloids and Pyridines</u>: Alkaloids (caffeine, nicotine, strychnine and quinine) inhibit nitrification at concentrations of 10^{-2} - 10^{-4} M. (Quastel and Scholefield, 1951). Toxic at similar concentrations as those for alkaloids are pyridine and its substitution products (Meikeljohn, 1954).

(iii) <u>Guanidine and Alkyl Carbamates (Urethanes)</u>: Acute inhibition of nitrification has been noted with the application of 10^{-6}M. concentrations of guanidine and ethylurethane (Quastel, 1946, 1951; Meikeljohn, 1954). Toxicity has also been observed for various substitution products of guanidine and urethane; the degree of toxicity being inversely related to the molecular weight of these derivatives (McBeath, 1962; Quastel and Scholefield, 1951). Ethylurethane, partially specific in its action, primarily inhibits the <u>Nitrosomonas</u> <u>spp</u>. (Quastel, 1946). The latter compound, like guanidine, is degraded after approximately twenty days; the nitrogen content appearing as nitrate. In comparing structural and inhibitory features of guanidine $(NH_2 \diagdown \overset{NH}{\underset{C}{\|}} \diagup NH_2)$, thiourea $(NH_2 \diagdown \overset{S}{\underset{C}{\|}} \diagup NH_2)$ and urethane $(NH_2 \diagdown \overset{O}{\underset{C}{\|}} \diagup OR)$, Quastel and Scholefield (1951) concluded that inhibition was likely associated with the chelating ability of the nitrogen atom adjacent to the carbonyl carbon atom. Anomalies such as the inactivity of acetamide

and urea would suggest a more complex mechanism of inhibition.

(iv) <u>Quinones</u>: The bacteriostatic action of certain quinones is well known and thus the inhibition of nitrification by hydroquinone (o-dihydroxybenzene), catechol (p-dihydroxybenzene) and Beta-napthaquinone-sulphonate at 10^{-4}M. concentrations is not unexpected (Lees, 1946). Inhibition of nitrification by quinones may involve one or more of the following general mechanisms of toxicity associated with these compounds (Hoffman-Ostenhof, 1963).

(1) The inhibition of sulfhydryl and amino dependent enzymes possibly through oxidation of these reactive groupings.

(2) Inactivation of coenzyme A.

(3) Uncoupling of oxidative phosphorylation.
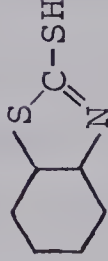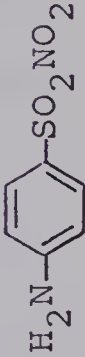
(c) <u>Nitrogenous Sulfur - Containing Organics</u>: The most powerful nitrification inhibitors include a number of nitrogenous organo-sulfur compounds exemplified in Table 1.

Within the groups of compounds represented by thiourea, isothiocyanates and dithiocyanates, activity decreases with increasing molecular weight (Tomlinson, 1966).

The capacity to strongly inhibit nitrification appears directly related to chelation properties of the compound

Table 1: Nitrogenous Organo-Sulfur Compounds Inhibitory to Nitrification

(Quastel & Scholefield, 1951; Jensen, 1952; Tomlinson, 1966)

Compound	Structural Formula	Effective Concentration
Thiosemicarbazides	$NH_2-\overset{\overset{S}{\|}}{C}-NH-NH_2$	10^{-6} M
Thiourea	$NH_2-\overset{\overset{S}{\|}}{C}-NH_2$	10^{-6} M
Thioacetamides	$NH_2-\overset{\overset{S}{\|}}{C}-CH_3$	10^{-6} M
Isothiocyanates	$-N=C=S$	10^{-5} M
2-Mercaptobenzothiazole	(ring structure) $\overset{S}{\underset{N}{}}C-SH$	10^{-5} M
Dithiocarbamates	$NH_2-\overset{\overset{S}{\|}}{C}-S-R$	$10^{-4}-10^{-5}$ M
Thiuroniums	$NH_2-\overset{\overset{NH}{\|}}{C}-SH$	$10^{-4}-10^{-5}$ M
Thiurams	$NH_2-\overset{\overset{S}{\|}}{C}-S-\overset{\overset{S}{\|}}{C}-NH_2$	$10^{-4}-10^{-5}$ M
Sulfanilamide Compounds	$H_2N-\!\!\!\bigcirc\!\!\!-SO_2NO_2$	$10^{-4}-10^{-5}$ M

(Lees, 1946). Several nitrogenous organo-sulfur compounds, specifically ethylxanthate, sodium diethyldithiocarbamate, salicylaldoxime and allylthiourea are known copper enzyme poisons. Their effect on nitrification may be reversed by minute additions of copper and to some extent, ferrous ions (Lees, 1946).

This correlation between chelation and antibacterial action has been clearly demonstrated. Studies with 8-hydroxy-quinoline, an active antibacterial agent, have shown that prepared isomers of this compound, known to be incapable of chelation, exhibit no antibacterial properties (Hewitt, 1963). Chelation is thought to promote inhibition of enzyme systems in at least two ways: (1) removal of essential metal cofactors (2) formation of a toxic metal-ligand complex. Chelated copper complexes are found particularily toxic. In the presence of Cu^{+2}, inhibition of Aspergillus niger is noted on introduction of 1 ppm. of a chelator, dimethyldithiocarbamate. Toxicity is attributed to the copper ion-ligand complex. At concentrations greater than 10 ppm. of the ligand, the established inhibion is reversed; conversion of the 1;1 (metal/ligand) complex to a non-toxic 2:1 saturated complex cited as the rationale. At ligand concentrations greater than 50 ppm., a third toxic phase is observed.

This effect was attributed to the inherent toxicity of
the ligand itself (Hewitt, 1963).

At concentrations of 10^{-3}M., the sulfur containing
amino acids; cystine, cysteine and methionine, notably
the latter, effectively retard nitrification (Jensen, 1952;
Quastel and Scholefield, 1951). The lowering of soil
pH on decomposition of these amino acids has been
suggested as a possible general mechanism of inhibition
(Quastel and Scholefield, 1951). The toxicity of methion-
ine, however cannot be explained so simply. Methionine
sulfoxide, detected as a decomposition product of
methionine, has been established as a competitive substrate
inhibitor in glutamate-glutamine transformations. Thus,
if condensation of NH_4^+ with glutamic acid is assumed
the first step in nitrification; methionine toxicity is
perhaps rationalized (Quastel and Scholefield, 1951).
Brown (1954), pursuing the question of methionine toxicity,
studied the action of various aklyl-sulfur-substituted
homocysteine (HS - $(CH_2)_2$ - $\overset{NH_2}{\underset{|}{CH}}$ - COOH) derivatives and
concluded that -SH (Mercapto) derivatives exhibited far
greater inhibition than $-\overset{}{\underset{\overset{\|}{O}}{S}}-$ (sulfoxide). Correspondingly,
sulfoxides were reported more effective than $-\overset{\overset{O}{\|}}{\underset{\overset{|}{O}(-)}{S}}-$

(sulfones). Hence evidence would indicate that the inhibitory effects of methionine and homologous amino acids in the process of nitrification cannot be ascribed primarily to their oxidation products.

(d) Electrolytes and Heavy Metals: Electrolytes such as the alkali metals inhibit nitrification although only at high concentrations ($10^{-1}M$.). Inhibition is also associated with the presence of rare elements e.g. indium, thorium, berylium (Tandon, 1968). More notable is the toxicity of ammonium, nitrite and nitrate to nitrification. Optimum concentrations of these substrates appear necessary for successful growth of the nitrifying chemautotrophs (Quastel and Scholefield, 1951; Aleem, 1957; Butts, 1960b).

Heavy metals such as Ph^{+2}, Cd^{+1}, Zn^{+2}, Mn^{+2}, Co^{+2}, Cu^{+2}, Ni^{+2}, Hg^{+2}, and Ag^{+2} significantly retard the rate of nitrification (Lees, 1946; Quastel and Scholefield, 1951; Tomlinson, 1966; Zavarzin, 1958). Particularly effective are Hg^{+2} and Ag^{+2} which inhibit at 10^{-5} to $10^{-6}M$. concentrations (Meikeljohn, 1954). Toxicity of heavy metals may be attributed to the consequences of chelation; namely the alteration of permeability barriers within an organism or the formation of toxic organometallics (Hewitt, 1963).

(e) <u>Metabolic Inhibitors</u>: A number of specific
enzyme inhibitors have been reported to retard nitrification
(Jensen, 1952; Lees, 1946; Quastel and Scholefield, 1951;
Butts, 1960a). Toxic as expected, are cytochrome uncouplers
such as cyanide and azide, phosphorylation inhibitors
such as dinitrophenol and iodoacetate as well as fluoride
(phosphatase inhibitor) and phenols.

(f) <u>Herbicides and Pesticides</u>: Various soil
sterilants methylbromide, pentachlorophenol, chloropicrin
(Andreae, 1963)   , fungicides dialkylthio-carbamates,
ethylenedithiocarbamates, chlorinated nitrobenzenes
(Rich, 1963; Casely, 1968) insecticides aldrin, parathion,
malathion, heptachlor, chlordane (Brown, 1954; Eno, 1958;
Shaw, 1960; Garretson, 1968) and herbicides 2,4D; 2,4,
5T; 2,2-dichloroproprionic acid; phenylureas; N-phenyl-
carbamates; acylanilides; chlorinated anilines (Jensen,
1952; Wilson, 1954; Hale, 1957; Thompson, 1969) have
been reported as inhibitory to nitrification. Concentra-
tions necessary to retard nitrification vary greatly,
chlorinated anilines being effective at 2-5 ppm. while
substituted thiocarbamates require up to 500 ppm. (Winely,
1969).

Researchers are generally of the opinion that field applications of common herbicides, pesticides and fungicides at current recommended rates, do little to significantly disturb the process of soil nitrification (Jensen, 1952; Wilson, 1954; Hale, 1957; Eno, 1958). Concern has been voiced with regard to the persistence and toxicity of degradation products (Thompson, 1969).

(B) Specific Inhibitors of Nitrification

(a) Inhibitors of Nitrosomonas Spp.: The fluoride ion, hydrazine, allythiourea and thiourea have been reported as compounds specifically inhibiting the conversion of ammonium to nitrite by autotrophic nitrifiers (Lees, 1946, 1963; Alexander, 1965). Hydrazine has been shown to specifically block the conversion of hydroxylamine to nitrite; allylthiourea and thiourea, copper enzyme poisons, the transformation of ammonium to hydroxylamine (Hoffman, 1953; Lees, 1963). A lengthened lag period prior to exponential growth has been observed for Nitrosomonas cells exposed to thiourea (McBeath, 1962).

(b) Inhibitors of Nitrobacter Spp.: 10^{-4}M. concentrations of cyanate and 10^{-5}-10^{-6}M concentrations of chlorate prove specifically toxic to Nitrobacter (Lees, 1946, 1963; Quastel and Scholefield, 1951). Cyanate is

thought to function as a substrate competitor and prevent
the access of nitrite to the surface of the oxidizing
enzyme (Butts, 1960a). Excepting at high inhibitor concen-
trations (10^{-3}M.), where cytochrome 551 inactivation has
been noted (Lees, 1963), chlorate exercises little influence
upon nitrite oxidation in soil enriched in Nitrobacter spp.
(Quastel and Scholefield, 1951). Low concentrations of
chlorate thus appear to block cell proliferation.

6. Nitrification Inhibitors - Significance to Agriculture

The agronomic use of non-phytotoxic chemicals capable
of selectively inhibiting the nitrifying bacteria has
been considered as a means of diminishing soil nitrogen
losses following fertilization. A significant number of
such compounds have been patented for possible agronomic
use; halogenated and nitro-phenols, hydrazine salts,
halogenated nitroanilides, bromo- and chloro- substituted
anilines, halogenated and amino pyridines (Alexander, 1965).
To date no product suitable for wide-spread agricultural
use has been discovered. Dicyandiamide (Reddy, 1964),
2-amino-4-chloro-6-methylpyrimidine (Patrick, 1968) and
2-chloro-6-trichloromethylpyridine (Goring, 1962 a,b),
particularily the latter, have received serious considera-
tion.

Chemical characteristics of and biological response
to 2-chloro-6-trichloromethylpyridine (N-Serve) have
been reported by Goring (1962 a,b). Non-phytotoxic at
normal rates of application, this chemical, administered
at concentrations of 0.5-20.0 ppm. using acetone as the
carrier solvent (solubility at 86°C - 194 gm./100gm
solvent), markedly inhibits the specific conversion
of ammonium to nitrite in soil for periods, reportedly,
as long as six weeks. Although independent of the clay
content of soil, the activity of N-Serve is adversely
affected by increased levels of soil organic matter and
PH. Under field conditions, partial control of the
nitrification of ammonium sulfate, ammonium nitrate,
ammonium phosphate, urea and aqua ammonia treated with
N-Serve has been reported (Goring, 1962b; Turner, 1962).
With the concentration of inhibitor expressed as a
percentage of the fertilizer nitrogen, 2% N-Serve was
needed for urea and ammonium salts in broadcast applications,
0.125% for band applications and 0.125%-0.5% was necessary
for aqua-ammonia.

The literature documenting the performance in field
trials of 2-chloro-6-trichloromethylpyridine is extensive
(Sweezy, 1962; Turner, 1962; Gasser, 1964, 1965, 1967,
1968; Prasad, 1966; Nowakowski, 1967, 1968; Hoflich, 1968;

Januet, 1968; Nishihara, 1968; Sabey, 1968). The ability

of N-Serve to retard nitrification and promote the

conservation of applied nitrogen is undisputed. Increased

phosphate uptake (Nielsen, 1967), decreased plant nitrate

content (Nowakowski, 1967) and claims as to increased

cotton, corn and sugar beet yields (Sweezy, 1962) have

been additional effects attributed to the use of N-Serve.

The inhibition of nitrification by N-Serve at the

pure culture and cell-free levels has been examined by

Campbell and Aleem (1965 I,II). 2-chloro-6-trichloromethyl-

pyridine has been shown to be a potent, specific inhibitor

of the ammonium oxidase system of Nitrosomonas 65%-70%

inhibition being realized in the presence of 0.5-1.5 ppm.

of the chemical. In contrast, the hydroxylamine cyto-

chrome system of Nitrosomonas was found to remain

uninhibited even when subjected to 100 ppm. N-Serve.

Likewise, the nitrite cytochrome oxidase system and the

nitrite cytochrome C reductase system of Nitrobacter

were noted to resist significant inhibition by N-Serve

until concentrations of 80-175 ppm. were introduced. Even

at such high concentrations, only 50% inhibition of

cytochrome oxidase system was observed; the nitrite

cytochrome reductase system remaining unaffected. Signifi-

cantly, addition of 6.0×10^{-4} M. Cu^{++} completely reversed

the N-Serve inhibited cytochrome oxidase of <u>Nitrosomonas</u>.
Inhibition by allylthiourea (10^{-6}M.) could not be
reversed in this manner.

SUMMARY OF THE LITERATURE REVIEW

Controversy continues to exist as to the relative merits of ammonium versus nitrate nitrogen in plant nutrition. Use of either the anionic or cationic nitrogen form appears to profoundly influence the uptake by plants of other macro- (K^+, $H_2PO_4^-$, $SO4^{--}$) and micro- nutrients as well as affecting soil pH (Viets, 1965).

The possible demerits of ammonium as a nitrogen source for plants is largely minimized when ammonium is considered in terms of fertilizer nitrogen economy and efficiency. Bound to the cation exchange complex in soil, ammonium resists downward leaching and removal from the rooting zone of plants, a fate common to nitrate. The problem of volitile N losses due to denitrification is also avoided by maintaining nitrogen in a reduced form. The controlled inhibition of nitrification is thus agronomically desire- able.

Intrigued by the initial observation that autotrophic nitrifying organisms appear inhibited by organic carbon substrates, and prompted by the desire to elucidate the biochemical pathways of nitrification, researchers have discovered and documented a wide array of compounds capable of inhibiting nitrification. Simple organics such as

peptone, nitrogenous organics, nitrogenous sulfur-containing
organics, electrolytes and heavy metals together with
common metabolic inhibitors have demonstrated varying
degrees of inhibitory activity. Organic compounds with
nitrogen and sulfur groupings attached to the same carbon
atom appear particularily toxic (Downing, 1964).

To date, nitrification inhibitors suitable for wide-
spread agricultural use are lacking. Dicyandiamide
(Reddy, 1964), 2-amino-4-chloro-6-methylpyrimidine
(Patrick, 1968) and 2-chloro-6-trichloromethylpyridine
(Goring, 1962 a,b), particularily the latter have received
considerable attention in this respect. Non-phytotoxic
at effective rates of 0.5-20.0 ppm., N-Serve has been shown
to selectively inhibit the transformation of ammonium to
nitrite in soil (Goring, 1962 a,b). The performance of
N-Serve in numerous field trials has resulted in unanimous
agreement as to its ability to retard nitrification.
However, its relatively short effective life-time (6 weeks),
its need for acetone as a carrier solvent and the fact
that it is a chlorinated hydrocarbon pose serious problems
with regard to expanded agricultural usage.

III.   MATERIALS AND METHODS

1.   Materials

(A)   Test Compounds

In the course of this study, seventy compounds were specifically examined and assessed for their potential as agriculturally applicable nitrification and/or ammonification inhibitors. Of this number, eighteen were organo-nitrogen formulations, twenty-three organo-nitrogen-organo-sulfur, twenty-nine were various polymeric derivations, including those of thiourea-formaldehyde.

Test compounds (Appendix I) were supplied by the Research Council of Alberta, Products Testing and Development Branch. A number of the chemicals so obtained were commercial preparations. The majority, however, were synthesized and forwarded by the Research Council of Alberta laboratories.

(B) The Thiourea-Formaldehyde Polymers

(a) Synthesis: In abbreviated terms, *synthesis of the active thiourea-formaldehyde polymeric derivatives,

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*Personal communications with Dr. M. Worsley
 Research Council of Alberta

required refluxing of a six fold excess of thiourea in a hydrochloric acid medium with formaldehyde. Heating was continued for one-half hour at which time the reaction was quenched with cold water. The ensuing precipitate was then filtered and dried. Relative molecular weights were approximated by comparisons of product solubility in hot water. More exacting molecular weight determinations were to employ the use of vapor pressure osmometric techniques.

(b) Product Description: Features of the thiourea-formaldehyde polymers as a class of compounds are found in Table 2.

(C) Soil Materials:

Laboratory and greenhouse experiments were conducted employing a Malmo clay loam soil sampled from the University of Alberta farm located at Ellerslie, Alberta. Physical and chemical characteristics of this soil appear in Appendix II. Two hundred to three hundred pounds of the clay loam were transported to the University of Alberta greenhouses where the soil was screened (-9 mesh) and air-dried. Washed sand (-9 mesh) was then mixed with the air-dried soil in a ratio of 1:1. The sand-loam mixture was thoroughly leached with tap water to remove accumulated nitrates, air-dried, screened (-9 mesh) and finally stored

Table 2: Descriptive Properties of RCA 118-66-IC

Physical Properties: Stable, amorphous, white powder

Chemical Structure: Monomer, Dimer or Trimer of

Thiourea & Formaldehyde

$$le \quad -(CH_2-NH-\underset{\underset{S}{\|}}{C}-NH)_{1-3}$$
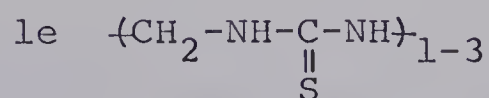
Solubilities: H_2O @ 30°C: 30% solubilized

H_2O @ 100°C: 80-90% solubilized

Acetone: insoluble

Ethanol (95%): insoluble

Dimethylsulfoxide: soluble

Dimethylformamide: soluble

Vapor Pressure: @ 20°C = NIL

in plastic bags for future use. The stored soil was
sampled periodically and assessed for nitrate-nitrogen
content. As expected, the level of the nitrogen form in
the prepared soil mixture was found to be negligible and
constant.

2. Methods

    (A) Qualitative Nitrate, Nitrite, and Ammonium Ion
        Tests

Presence of the ammonium ion was detected using
Nessler's Reagent as an indicator solution. A positive
reaction for the presence of the ammonium ion was marked
by the appearance of a yellowish-orange precipitate.

The nitrite was detected employing Tromsdorf's
Reagent (Appendix III) in an acid medium (1:3 dilute
sulfuric acid). In the presence of the nitrite ion, a blue-
black coloration was imparted to the indicator solution.

Diphenylamine solution (Appendix III) in an acid
medium (concentrated sulfuric acid) was used to qualita-
tively assay for the nitrate ion in the indicator solution.
As this test reacts positively with the nitrite ion also,
it was necessary to discount the presence of the latter ion
prior to attempts to detect the nitrate ion.

(B) <u>Quantitative Assay Methods for the Nitrate Ion</u>:
    <u>the Nitrate Electrode</u>

A membrane electrode (Model 92-07), specific in
response to the nitrate ion activity, has been developed
by Orion Research Incorporated of Cambridge, Massachusetts.
The electrode develops a potential, detectable on the
expanded millivolt scale of a pH meter, which is propor-
tional to the log of the activity of the nitrate ion in
solution. Coupled with a reference electrode e.g. calomel,
this instrument suggested both a simple and rapid means of
performing quantitative nitrate determinations.

Several workers (Myers and Paul, 1968; Mahendrappa,
1969) have investigated the nitrate electrode and have
reported its performance to be satisfactory.

In our exploratory studies (Appendix IV) however,
continual instrument drift, inability to obtain satisfactory
reproduceability and marked electrode sensitivity to
interference by other ions and organic matter, prompted an
abandonment of this instrument in favor of the established
phenoldisulfonic acid method of quantitative nitrate
ion determination.

(a) <u>The Phenoldisulfonic Acid Method</u>: Initially,
various methods were considered for the quantitative
determination of nitrate in soil extracts. In a brief

literature survey, the following possible procedures
were revealed:.

(1) Reduction of nitrate, followed by the determination
of nitrite (Nelson, 1954) and ammonia (Richardson, 1938).

(2) Colorimetric measurement of a nitration product
formed with reagents such as: brucine (Fischer, 1958;
Robinson, Allen and Gacoka, 1959; Baker, 1967), chromotropic
acid (Clarke and Jennings, 1965) 2, 4-xylenol (Buckett, 1955),
and phenoldisulfonic acid (Easthoe and Pollard, 1950;
Bremner, 1956)

(3) Polarography (Skyring, Gary and Skerman, 1958;
Malingerove, 1964).

(4) Ultra-violet Spectroscopy (Cause, 1967).

Being proven, well-defined and widely accepted, the
phenoldisulfonic acid method of quantitatively determining
nitrate-nitrogen concentrations in soil extracts was
selected for use. The results of a quantitative nitrate
assay for treatment and control soil samples were
assessed on a relative basis i.e. detected nitrate levels
for the treatments were compared with that of the control
and then analysed for significance. Each set of analyses
was thus complete in itself. As such it was reasoned that
as long as consistency with regard to the accuracy of
the nitrate assay was maintained, certain procedures

suggested to insure a high degree of recovery and detection could be sacrificed in the interests of economy in terms of time, labor and chemicals. The procedures outlined by Bremner (1965) for the phenoldisulfonic acid method of quantitatively determining nitrate-nitrogen were thus modified to suit these objectives.

(i) Extraction: Soil samples were transferred by washing from the incubation containers to 500 ml. erlenmyer flasks. Sufficient distilled water was added to bring the total volume of water in each flask to 250 ml. Flasks containing the soil-water slurry were placed on a rotary mechanical shaker and agitated for 15 minutes. Approximately 2 gm. of $Ca(OH)_2$ was then added to each flask and agitation was resumed for an additional 15 minutes. The flasks were removed from the shaker and left for one hour to allow flocculation and settling of the soil particles. 25 ml of the resulting clear supernatant were pipetted into an evaporating dish. The evaporating dishes were placed in a drying oven set at 90°F. Samples, evaporated to dryness, were then subjected to the phenoldisulfonic acid treatment for quantitative nitrate-nitrogen assessment.

(ii) Analysis: Standard nitrate solutions were prepared and analysed in the manner described below for the range 0-550 micrograms of nitrate-nitrogen. The

resulting standard curve appears in Appendix V.

Samples, evaporated to dryness, were allowed to cool.
4 ml of phenoldisulfonic acid were added to each sample and
allowed to react for a period of 10 - 15 minutes. Where
necessary, a teflon stirring rod was used to loosen the
precipitate. The contents of each evaporating dish was then
transferred to 200 ml volumetric flasks. The flasks were
partially filled with distilled water and agitated to bring
the contents into solution. 25 ml of 8.0 M KOH solution
were pipetted into each flask. To ensure maximum color
development, the alkalinity of the solution resulting was
periodically checked. Using distilled water, the contents
of each volumetric were brought to volume. Colorimetric
assays were performed with a Klett-Summerson Photoelectric
Colorimeter equipped with a 420 (blue) filter. Colorimetric
readings were converted directly into micrograms of nitrate-
nitrogen by referring to the prepared standard curve.

(C) Laboratory Studies

(a) Routine Incubation Procedures: The capacity to
inhibit nitrification and/or ammonification was determined
by incubation tests in which a reduced nitrogen source,
urea, was added to soil in the presence of selected test
compounds. The degree of inhibition was gauged by com-
paring, quantitatively, the formation of nitrate in the

presence and the absence of such inhibitor compounds.

Desired test compounds were applied in solid or solution form to 50 gm of the previously described soil mixture placed in sealable glass tumblers. Treatments were carried out in triplicate. After mixing thoroughly, urea, as an aqueous solution, was added to each treatment at a rate of 200 ppm. nitrogen. The moisture content of the soil in each treatment was adjusted with distilled water to that approximating two-thirds field capacity. The loosely sealed containers were then placed in a controlled environmental chamber, void of light, held at a relative humidity of 98% and thermostated at 32°C. The incubation containers were opened periodically to allow aeration. After 21 days, each sample was quantitatively analysed for accumulated nitrate-nitrogen.

Arrangement of the treatments within the incubation chamber was that of a simple randomized design.

(b) Pure Culture Studies: An alternate method of screening suspected anti-nitrification and/or anti-ammonification compounds was attempted employing a mixed, liquid culture of nitrifiers. A growth medium (Appendix VI) of mineral salts featuring ammonium as the nitrogen source was prepared and dispensed in 20 ml aliquots into

sterile, cotton-plugged 200 ml erlenmeyer flasks. Innocula-
tion of the medium was followed by the addition of an
established concentration (0.5-10.0 ppm.), of the test
compounds. Treated and untreated cultures were incubated in
darkness at 20°C with periodic agitation to ensure adequate
aeration. After one week, qualitative nitrite and nitrate
tests were performed at periodic intervals. The outlined
procedure was repeated for successively higher concentrations
of the added inhibitor until a threshold concentration
limiting the further formation of nitrate was observed.

   (c) An Examination of Inhibitory Activity as a
       Function of Inhibitor Concentration: For selected
test compounds, inhibitory activity was studied as a
function of the application rates. Examined was the per-
formance of inhibitors at concentrations ranging from
0-180 ppm in the presence of 200 ppm nitrifiable nitrogen
applied as urea. Incubation and assay procedures were
observed as previously outlined.

   (d) An Examination of Nitrogen Sources and their
       Influence upon the Inhibitory Activity of Thiourea-
       Formaldehyde Polymer 118-66-IC: A preliminary
experiment was undertaken in an attempt to determine where,
in the sequence of reactions associated with ammonification
and nitrification, the inhibitory action of 118-66-IC
was directed. This problem was approached through

routine laboratory incubation tests in which three nitrogen sources were provided at a rate of 100 ppm applied nitrogen. The selected nitrogen sources were urea, ammoniun chloride, and sodium nitrite. The thiourea-formaldehyde polymer was applied in powder form at a rate of 400 ppm.N-Serve (2-chloro-6-trichloromethylpyridine), included in this experiment for comparative purposes was applied as a solution in acetone at a rate of 100 ppm. The high rate of inhibitor application was purposely established to accentuate and hopefully, clearly reveal the point(s) of inhibition.

(D) Greenhouse Investigations

(a) Routine Phytotoxicity Studies: For those nitri-fication and/or ammonification inhibitors displaying encouraging potential in incubation tests, greenhouse trials were conducted to assess their phytotoxic properties. To this end, the growth response of plants in the presence and absence of these tests compounds and also in the presence of urea alone, was compared.

Treatments, in triplicate or quadruplicate at desired rates of addition, were applied in powder or solution form to 1000 gm of the prepared soil mixture potted in 5" diameter plastic containers. Mixing was accomplished with

the aid of a hand churn. The moisture content of the soil in each container was adjusted and maintained with distilled water to a level approximating field capacity. Each pot was then seeded, surfacially, with 1.0 gm of Reed Canary Grass seed. Germination was initiated under the cover of moistened filter paper; the latter being removed on establishment of growth. A simple randomized experimental design was employed and growth under controlled environmental conditions (temperature: 22°C, photoperiod - 16 hours) was allowed to continue until plant maturity and establishment of stable growth conditions.

Retardation of germination, germination toxicity and evidence of chlorosis or nicrosis were features to be noted.

(b) Specific Studies Concerning the Thiourea-Formaldehyde Polymer 118-66-IC:

(i) Plant Response Studies: An attempt to correlate the growth response of Reed Canary grass to varying application rates of 118-66-IC and urea was carried out. Urea nitrogen concentrations of 100-200 ppm were supplied and supplemented with concentrations of 118-66-IC nitrogen such that the level of total nitrogen supplied was 200 ppm.

A simple randomized experimental design incorporating
5 replicates of 14 treatments was employed. Previously
outlined greenhouse procedures with the following modi-
fications were followed. After 21 days of incubation
and prior to seeding, each treatment was leached with
1000 mls of distilled water to remove accumulated nitrate.
At appropriate intervals, the growth was harvested oven-
dried at 70°C and assessed for yield of dry matter.

(ii) Longevity Studies: For compound 118-66-IC, an
attempt was made to establish the period of effective
activity. With this aim, an experiment involving seeded
treatments of 118-66-IC and urea applied at varying
concentrations was cropped to the point of nitrogen
exhaustion and maintained under potential growth condi-
tions for a further period of ten months. The soil from
each individual treatment was screened to remove plant
debris, repotted, leached with distilled water to remove
soluble nitrates and regenerated with urea nitrogen at a
rate of 200 ppm. Following a two week incubation period,
soil cores were removed from each treatment and a 10 gm
sub-sample was assessed quantitatively for newly-formed
nitrate nitrogen. To discount the possibility that
phytotoxic elements may have accumulated during the period
of fallow storage, regenerated treatments were seeded

to Gateway barley and subsequently observed.

(iii) <u>Performance Study of Inhibitor-Coated Urea</u>: Five compounds showing potential as inhibitors of nitrification and/or ammonification were forwarded to the Research Council of Alberta laboratories to be employed as a coating for urea granules. Specifications of the manufactured product appears in Appendix VII.

Incubation tests with the coated urea were performed under greenhouse conditions (temperature: 22°C,relative humidity: ambient, photoperiod: 16 hours). The manufactured product was applied to 1000 gm of the potted prepared loam-sand mixture at a rate such that 200 ppm nitrogen was supplied to each treatment. The moisture content of the soil was adjusted to and maintained at approximately two-thirds field capacity. At intervals of two and four weeks, soil cores were removed from each container, pooled and sub-sampled. Quantitative nitrate nitrogen analyses were carried out on the 10.0 gm sub-samples.

(c) <u>Long-Term Incubation Studies with Refractive High Molecular Weight Thiourea-Formaldehyde Polymers</u>: A thiourea-formaldehyde polymer (27-3), tested and found refractive as an inhibitor of nitrification and/or ammonification, was applied at a rate of 100 ppm. to 2000 gm of the potted prepared loam-sand mixture in an attempt

to investigate the possibility that compound degradation
i.e. cleavage of the polymer chain, may give rise to
inhibitory activity. Treated and control soils were
maintained under greenhouse conditions at 2/3 field cap-
acity, unseeded and in a well-aerated state for eight
months. At monthly intervals 50 gm duplicate samples
of the treated and untreated soil were removed, placed in
glass tumblers, supplied with 200 ppm nitrogen as urea
and subjected to routine laboratory incubation procedure.

(E) Field Studies:

Exploratory field trials were initiated in the fall of
1966 with an active low molecular weight thiourea-
formaldehyde polymer preparation. The field plots, located
at the University of Alberta farm at Ellerslie, Alberta;
were constructed on unirrigated, Eluviated Black
Chernozemic soil seeded to pasture grasses. The thiourea-
formaldehyde treatments were included as part of a much
larger field experiment based on a randomized block
design incorporating four replicates.

The thiourea-formaldehyde polymer was suspended in
distilled water and applied as a slurry at rates of 200
and 1000 lbs/acre. Unamended and inhibitor grass plots
received no further special attention. With the aid of
a garden-sized forage harvester, mid-summer and fall

cutting of the mature grass was conducted to initiate fresh growth. Evidence of phytotoxicity, visual differences in growth, degree of regrowth following harvesting, vigor and color of the grasses were features assessed for comparison throughout the course of this experiment.

In May of 1969, soil samples were removed from the thiourea-formaldehyde plots which had been maintained for two and one-half years. In a similar manner, the (unamended) check plots were sampled. The soil was transported to the laboratory in plastic bags, dried, screened (-9 mesh), weighed into 50 gm portions, placed in glass tumblers, supplied with 200 ppm urea nitrogen and subjected to routine laboratory incubation procedures.

IV. RESULTS AND DISCUSSION

1. Aspects Concerning Experimental Procedures

(A) Methods of Studying Soil Nitrification

Soil incubation and soil perfusion techniques have
both been employed in the study of soil nitrification.
The choice of methodology has largely been governed by
the objectives of the investigation. With the perfusion
technique, in which a soil sample is intermittently but
frequently perfused with an aerated solution of
nitrifiable material, a soil enriched with a nitrifying
population is established and maintained under controlled
conditions of moisture, aeration and nutrient supply. Soil
in such a state of bacterial enrichment behaves like a
biological system and as such, kinetic and biochemical
studies on soil processes such as nitrification are possible
with accurately reproducible results (Lees, 1946). From
an agronomic standpoint, however, the soil perfusion
technique presents definite disadvantages. Bacterially
enriched soil, by design, possesses an abnormally distributed
microbial population. Thus products studied by soil
perfusion techniques are not exposed to a truely represen-
tative soil microflora and considerations such as the
nature and consequences of product degradation cannot be

satisfactorily assessed. Soil incubation techniques, however, involve the proliferation of native soil micro-organisms. Exposure of soil amendments to a mixed microbial flora more closely approximating field conditions is thus anticipated.

With regard to this project, the nature of many of the chemical compounds considered as potential ammonification and/or nitrification inhibitors further discouraged adoption of the soil perfusion technique as a screening procedure. Being insoluble in most of the common solvents, such chemicals could be assessed for potential only by soil incubation methods.

(B) Quantitative Methods

Quantitative determinations of nitrate-nitrogen in soil, as recommended by Bremner (1965), involve an initial extraction of the soil sample with an aqueous, saturated $CaSO_4$ solution; the calcium ion initiating soil floccula-tion. In a subsequent step, the addition of $CaCO_3$ to aliquots of the filter clarified soil extract is suggested in order to render the solution basic. Under such pH conditions, the loss of NO_3^- is prevented during the process of heating and evaporation to dryness. For this study, the foregoing objectives were conviently combined into a

single step by employing an extracting solution of $Ca(OH)_2$ (11.6 M). The influence of these modifications upon recovery and quantification of nitrate nitrogen is shown in Table 3. 98% recovery of the total theoretically available nitrate nitrogen was consistently realized. The phenoldisulfonic acid method for quantitatively determining nitrate nitrogen is adversely influenced by the presence of organic matter and nitrite ions (Bremner, 1965). Additional data presented in Table 3 point to similar conclusions.

In evaporating aliquots of the clarified soil extract to dryness, the use of a steam bath is recommended by Bremner (1965), in order to prevent possible charring of organic matter present and subsequent interference of color development. Although a drying oven was employed in this study, adverse results were noted only when prolonged heating was carried out (Table 4). In such cases, a slight but consistent loss of nitrate nitrogen was detected.

Quantitative nitrate nitrogen assays carried out on unamended field samples of Malmo clay loam showed a significant accumulation of nitrate nitrogen (Table 5). The expected difference in accumulated nitrate levels between fallow and cropped

Table 3:   Effect of Nitrite Ions & Organic Matter

Upon Nitrate Nitrogen Detection by the

Phenoldisulphonic Acid Method

Concentration of Nutrient Broth in Reaction Volume	Concentration of Nitrite Ion in Reaction Volume	Total Available Nitrate-N (µg)	Recovered Nitrate-N (µg)
0	0	100	98
0	0	100	98
0	0	100	98
5%	0	100	69
5%	0	100	70
5%	0	100	73
0	0.6 ppm	100	90
0	0.6 ppm	100	89

Table 4: Effect of Prolonged Heating & Evaporation at

90°C upon Nitrate Nitrogen Detection by the

Phenoldisulphonic Acid Method

Period of Heating (Hrs.)	Total Available Nitrate-N (µg)	Recovered Nitrate-N (µg)	% Recovery of Nitrate-N
[1]4	150	146	97
4	150	148	99
4	150	146	97
[2]16	150	138	92
16	150	138	92
16	150	138	92

[1]A period of heating sufficient to evaporate a soil extract aliquot to point of dryness.

[2]An arbitrarily selected period of prolonged heating of the soil extract aliquot.

Table 5: Background Levels of Nitrate Nitrogen in
          Selected, Leached & Unleached, Unamended
          Soil Samples (50 gm.).

Soil Description	Treatment	Concentration Nitrate Nitrogen ($\mu g/gm$)
[1]Mo.CL. (fallow)	Unleached	34
Mo.CL. (fallow)	Unleached	29
Mo.CL. (fallow)	Unleached	34
Mo.CL. (cropped)	Unleached	7
Mo.CL. (cropped)	Unleached	6
Mo.CL. (cropped)	Unleached	7
[2]Mo.SiCL. (cropped)	Unleached	20
Mo.SiCL. (cropped)	Unleached	20
Mo.SiCL. (cropped)	Unleached	21
Mo.SiCL. (cropped)	Unleached	19
Mo.SiCL. (cropped)	Leached	5
Mo.SiCL. (cropped)	Leached	5
Mo.SiCL. (cropped)	Leached	3
50/50(V/V)Mo.CL. & Sand	Unleached	10
50/50(V/V)Mo.CL. & Sand	Unleached	10
50/50(V/V)Mo.CL. & Sand	Unleached	9
50/50(V/V)Mo.CL. & Sand	Leached	1
50/50(V/V)Mo.CL. & Sand	Leached	1
50/50(V/V)Mo.CL. & Sand	Leached	2

[1]Malmo Clay Loan   [2]Malmo Silty Clay Loam

soils was also evident. Water-leached samples of soil,
however, showed desired low nitrate nitrogen levels.
Storage of the leached, air-dried, sieved soil did not
alter the initial low background levels of nitrate nitrogen.

2. An Assessment of the Screening Procedure Employed
   for Chemical Compounds Selected as Potential Nitrifi-
   cation Inhibitors

   (A) Studies Employing Purified Cultures of Autotrophic
       Nitrifying Bacteria

   The influence of thirty-one chemical compounds
upon the ability of autotrophic nitrifying organisms to
transform ammonium to nitrate was examined. Three categories
of chemicals were considered; nitrogen-containing organics,
sulfur and nitrogen-containing organics and polymeric deri-
vatives of thiourea. Data presented in Table 6
summarizes the results of this investigation.

   Chelation and removal of the copper ion, which is
suspected as a prosthetic group in the ammonia oxidizing
system has been suggested as a possible mechanism for the
inhibition of nitrification (Lees, 1946; Quastel and
Scholefield, 1951; Lees, 1955; Campbell and Aleem, 1964).
An interpetation of the results of Table 6 on the basis
of this theory, shows a satisfactory agreement between

Table 6: Concentrations of Selected Chemical Compounds

Required to Totally Inhibit Ammonium Oxidation

in Purified Cultures of Autotrophic Nitrifying

Bacteria

Chemical Compound	Concentration Required to Produce 100% Inhibition of Nitrification (ppm.)
Nitrogen-Containing Organics	
bis(salicyaldehyde)ethylenediimine	< 1.0
2-nitroso-1-napthol	>12
N-nitrosodimethylamine	>20
N-nitrosodiethylamine	>16
hexamethylenetetramine	>12
1,2-diamino-N,N'-bis(methyl-2-hydroxy-5-nitrobenzyl)ethane	< 2
diacetylmonoximenitrophenylhydrazone	< 4
benzoinoxime	< 3
1,2-diamino-N,N'-bis(methyl-2-hydroxy-5-methylbenzyl)ethane	< 0.5
2-pyridinealdoxime	>12
N-(2-hydroxy-5-methylbenzyl)-N,N-(dimethyl)amine	< 1.0
Nitrogen & Sulfur-Containing Organics	
Thiosemicarbazide	>14
α-Mercaptopyridine	>12
bis(diethylthiocarbamyl)disulfide	>10
2-Mercaptobenzothiazole	< 2
β-Phenylthiosemicarbazide	< 4
2-Thiouracil	< 1
2,5-Dimercapto-1,3,4-thiadiazole	< 2

Table 6 (Continued)

Chemical Compound	Concentration Required to Produce 100% Inhibition of Nitrification (ppm.)
N(2-mercapto-5-methyltolyl)-N,N-dimethylamine	< 1.0
N(1-methoxybutyl) thiourea	>10
N(1-hydroxybutyl) thiourea	< 0.5
N(1-hydroxybutyl) thiourea	< 0.25
Dithiooxamide	>12
1,2-diamino-N,N'-bis(methyl-2-mercapto-5-methylbenzyl)ethane	>12
Thiouric acid	< 1.0
Polymeric Derivatives of Thiourea	
Thioureaformaldehyde polymer	< 2.0
Urea-thiourea copolymer	< 1.0
Thioureaformaldehyde polymer (high molecular weight)	>10
Phenol-thiourea copolymer	>12
Thiophenol-thiourea copolymer	> 8.0
Butraldehyde-formaldehyde-thiourea polymer	< 2.0

the former and experimental data.

Of the nitrogen-containing organics,
bis(salicyaldehyde) ethylenediimine, 1,2diamino-N N'-bis
(methyl-2-hydroxy-5-methylbenzyl) ethane and N(2-hydroxy-5-
methylbenzyl)-N,N'(dimethyl)amine were the most active,
causing 100% inhibition of nitrification at concentrations
below 1 ppm. Examination of the respective chemical
structures shows the chelation of divalent metal ions is
readily feasible (Figure 1).

In comparison to other compounds of Figure 1, N-nitro-
sodimethylamine was found to be only weakly inhibitory,
100% inhibition of nitrification occurring at concentrations
exceeding 20 ppm. The chemical structure of this compound,
illustrated in Figure 1, does not possess features amenable
to strong chelation.

1,2-diamino-N,N'-bis(methyl-2-hydroxy-5-methylbenzyl)
ethane and 1,2-diamino-N,N'-bis(methyl-2-hydroxy-5-nitro-
benzyl) ethane differ structurally only by the substituent
of the 5 position on the benzyl moiety. The exchange
of an electron donating alkyl group for an electron with-
drawing nitro group results in a decrease in ability to
inhibit nitrification. Examination of the possible
tautomeric structures for 1,2-diamino-N,N'-bis(methyl-2-

Figure 1: Non-Chelated & Chelated Chemical Structures of

Selected Nitrogen-Containing Organics

A. bis(salicyaldehyde)ethylenediimine
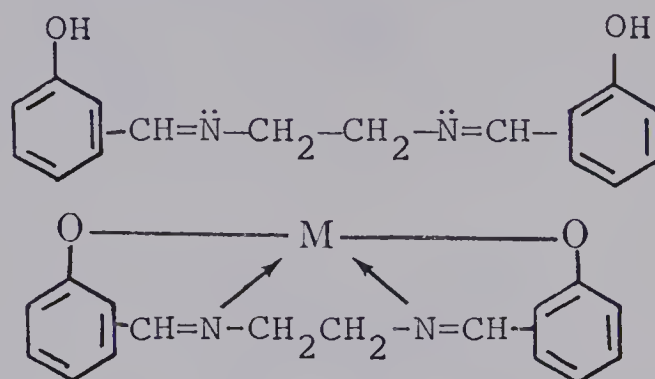
Hypothetical Chelation Structure

B. 1,2 Diamino-N,N'bis(methyl-2-hydroxy-5-methylbenzyl)ethane
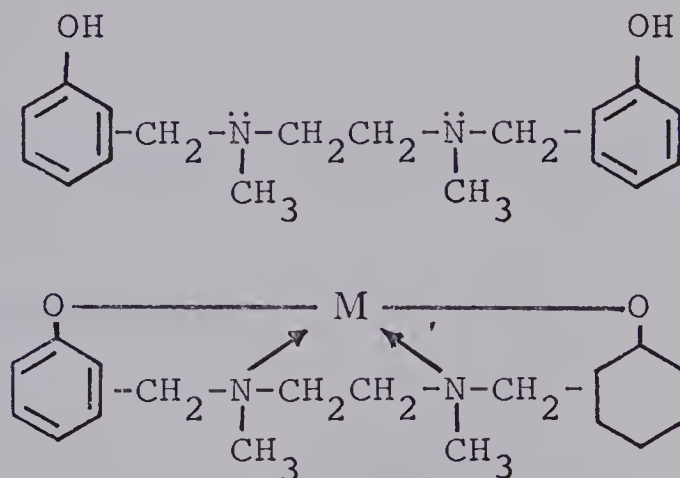
Hypothetical Chelation Structure

C.   N(2-hydroxy-5-methylbenzyl)-N,N-(dimethyl) amine

OH
$\overset{\displaystyle |}{}$

—CH$_2$—N$\overset{\displaystyle CH_3}{\underset{\displaystyle CH_3}{}}$

CH$_3$

CH$_2$—N$\overset{\displaystyle CH_3}{\underset{\displaystyle CH_3}{}}$

O

M

O

CH$_3$
N—CH$_2$
CH$_3$
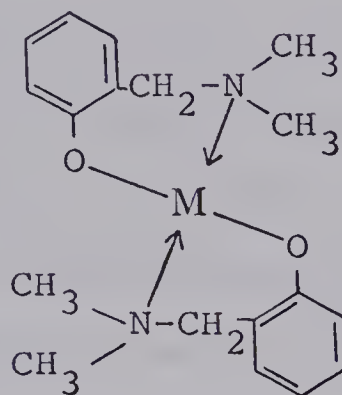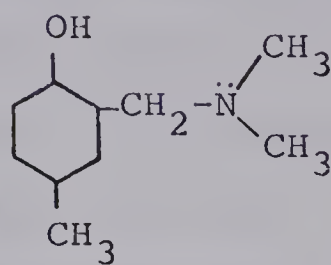
Hypothetical Chelation Structure

D.   N-Nitrosodimethylamine

O=N—N$\overset{\displaystyle CH_3}{\underset{\displaystyle CH_3}{}}$
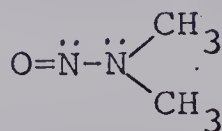
hydroxy-5-nitrobenzyl) ethane suggest a possible explanation

for this observation (Figure 2). In assuming the isomeric

configuration of IV, the structural features necessary

for strong chelation are lost; thus a decrease in the

ability to inhibit nitrification may be expected.

The effectiveness of sulfur and nitrogen - containing

organics as inhibitors of nitrification may also be

related to their ability to chelate. Examinatiion of the

chemical formula for N(1-hydroxybutyl)thiourea and

N(1-hyroxypropyl) thiourea, two organo-nitrogen and sulfur

containing compounds found to promote 100% inhibition

of nitrification at concentrations less than 0.5 ppm,

reveal ideal steric and structural coordination features.

Chemical compounds displaying relatively poor potential

as chelators e.g. dithiooxamide, thiosemicarbazide

proved correspondingly ineffective as inhibitors of nitrifi-

cation.

Notable is the difference in inhibitory activity

between N(1-hydroxybutyl) thiourea and N(1-methoxybutyl)

thiourea. By substituting a hydroxyl group in place of a

methoxy moiety in N(1-methoxybutyl) thiourea, inhibitory

activity was increased some 20 fold. Such an obervation

again may be attributed to changes in ability to chelate.

Figure 2: Hypothetical Isomeric Structures for

1,2-Diamino-N,N'-Bis(Methyl-2-Hydroxy-

5-Nitrobenzyl) Ethane
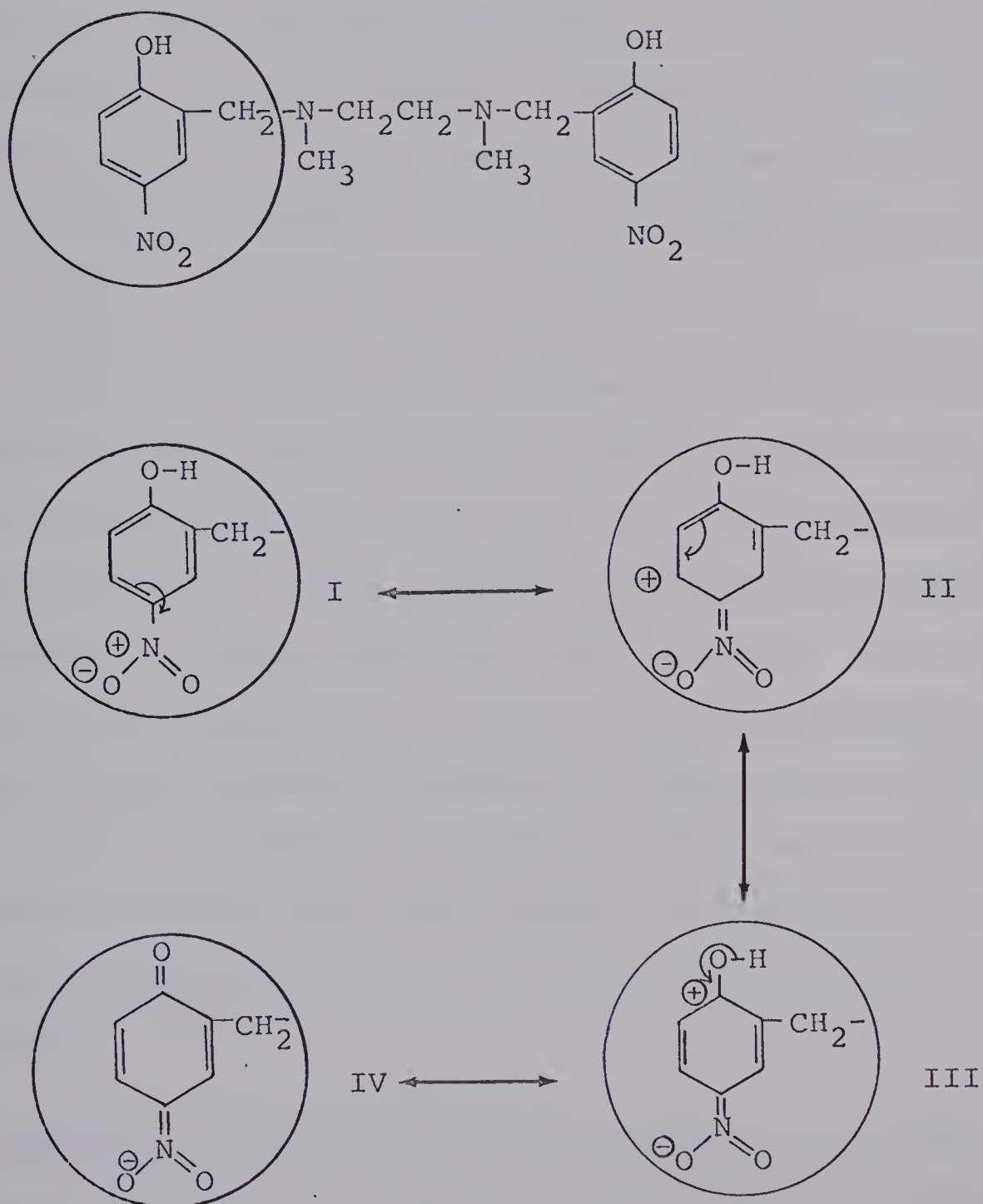
Among the nitrogen and sulfur containing organics,
exceptions to the noted correlation between the ability
to chelate and the effectiveness as an inhibitor of
nitrification exists. 1,2-diamino-N,N'bis(methyl-2-
mercapto-5-methylbenzyl) ethane, while structually
exhibiting ideal chelation features, proved relatively
ineffective as a nitrification inhibitor. An essentially
identical organo-nitrogen compound, 1,2-diamino-N,N'bis(methyl-
2-hyroxy-5-methylbenzyl) ethane was a highly effective
nitrification inhibitor.

Among the polymeric derivatives of thiourea, the low
molecular weight thioureaformaldehyde polymer and the
urea-thiourea copolymer performed well as inhibitors
of nitrification. Their chemical structures, as shown
in Figure 3, are also favorable for chelation. Signifi-
cantly, the high molecular weight thioureaformaldehyde
polymer was relatively inactive as a nitrification inhibi-
tor. Decreased product solubility as well as steric-
factors may account for this result. Sterochemical factors
may also explain the inactivity of the phenol-thiourea
copolymer.

Reports of the acute inhibitory effects of organo-
mercapto and organo-nitrogen and sulfur containing com-

Figure 3: Unchelated & Chelated Chemical Structures of
Selected Polymeric Derivatives of Thiourea

A. Thioureaformaldehyde polymer (low molecular weight)

$$
\begin{array}{ccc}
& S & S \\
& \| & \| \\
\{\ddot{N}H-C-\ddot{N}H-CH_2-\ddot{N}H-C-\ddot{N}H\}
\end{array}
$$

$$
\begin{array}{c}
S\text{———}M\text{———}S \\
\{N=C-NH-CH_2-NH-C=N\}
\end{array}
$$
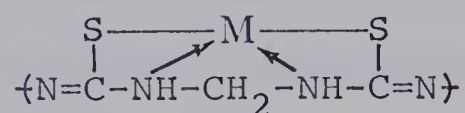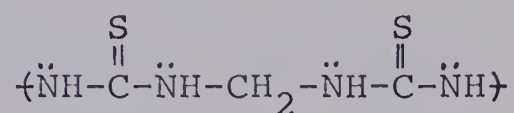
Hypothetical Chelation Structure

B. Urea-Thiourea Copolymer

$$
\begin{array}{ccc}
& O & O \\
& \| & \| \\
\ddot{N}H_2-C-\ddot{N}H-\ddot{N}A-C-\ddot{N}H_2
\end{array}
$$

$$
\begin{array}{c}
O\text{—}M\text{—}S \\
\ddot{N}H=C-NH-NH-C=\ddot{N}H
\end{array}
$$
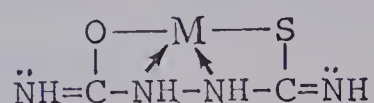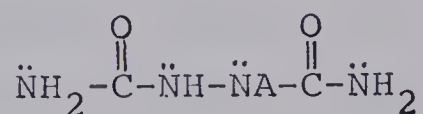
Hypothetical Chelation Structure

pounds upon nitrification are well documented (Quastel
and Scholefield, 1951; Jensen and Sorensen, 1952; Brown
et al, 1954; Downing et al, 1964). Similar findings
were noted in this study. The notable inhibitory activity
of several of the polymeric derivatives of thiourea,
however, is, to this writer's knowledge, a new and as yet,
unreported observation.

(B)   Soil Incubation Studies

The performance of chemical compounds, displaying a
high degree of inhibitory activity in studies under pure
culture conditions, were further examined by soil methods.
Arbitrarily, those compounds active at less than 1 ppm
were selected for study. The results of this investigation
are presented in Table 7.

Although concentrations of the inhibitors were
increased 10-80 fold the level of inhibitory activity
noted in pure culture studies was not obtained. Signi-
ficant loss of inhibitory activity was noted for all
compounds. N(1-hyroxybutyl) thiourea and N(1-hyroxypropyl)
thiourea, particularily active when tested in purified
cultures of autotrophic nitrifiers, exhibited a drastic
loss of ability to inhibit nitrification in a soil medium.
The loss in inhibitory activity suffered by the urea-

Table 7: A Survey of the Ability of Selected
Chemical Compounds to Inhibit Nitrification in Soil

Chemical Compound	[1]Concentration of Chemical Employed in "Pure" Culture Studies (ppm.)	Concentration of Chemical Added to 50 gm. of Soil (ppm.)	[2]% Inhibition of Nitrate Formation
Nitrogen-Containing Organics			
bis(salicyaldehyde)ethyenediime	1.0	10 / 20	74 / 82
1,2-diamino-N,N'-bis(methyl-2-hydroxy-5-methylbenzyl)ethane	0.5	10 / 20	77 / 86
N(2-hydroxy-5-methylbenzyl)-N,N-(dimethyl) amine	1.0	10 / 20	56 / 69
Nitrogen & Sulfur Containing Organics			
N(1-hydroxybutyl) thiourea	0.5	10 / 20	50 / 54
2-thiouracil	1.0	10 / 20	49 / 79
N(2-mercapto-5-methylbenzyl)-N,N-dimethylamine	1.0	10 / 20	43 / 70
Polymeric Derivatives of Thiourea			
Urea-thiourea copolymer	4.0	10 / 20	33 / 33
Urea (control)		0	0

[1]Chemical concentration required for 100% inhibition of nitrate formation in purified cultures of autotrophic nitrifiers

[2]% inhibition of nitrate formation $= 100 - \left\{ 100 \left[\dfrac{\text{Nitrate concentration in treatments i.e. urea \& inhibitor}}{\text{Nitrate concentration in control}} \right] \right\}$

thiourea copolymer in a soil environment was equally
notable.   Although a concentration of 20 ppm was required,
bis(salicyaldehyde) ethylenediime, 1,2-diamino-N,N'
bis(methyl-2-hydroxy-5-methylbenzyl) ethane and 2-thiouracil
inhibited nitrification in soil to a respectable degree.

Inhibitor compoun's added to soil may suffer non-
homogenous dispersion, degradation and inactivation by soil
microorganisms and inactivation by physical and chemical
adsorption to active soil constituents . Thus to
maintain in a soil environment, levels of activity noted
in experiments with purified cultures of autotrophic
bacteria, higher concentrations of chemicals will
necessarily be required. It is therefore apparent that
while testing of compounds inhibiting nitrification in
cultures of autorophic nitrifying bacteria provides a
rapid indicative survey of effectiveness, the true
capabilities of these compounds cannot be assessed until
testing is carried out in a soil medium.

3.   Chemical Derivatives of Thiourea as Potential
     Nitrification Inhibitors

   (A) Thiourea
   Thiourea has long been noted as a powerful inhibitor
of nitrification.   In the presence of 10^{-3} M - 10^{-6} M

thiourea, total inhibition of nitrification has been observed in pure cultures of <u>Nitrosomonas</u> <u>spp</u>.(Jensen and Sorensen, 1952) in activated sludge (Downing et al, 1964) and in soil perfusion systems (Quastel and Scholefield, 1949, 1951; Jacques, Robinson and Chase, 1959) Reportedly, thiourea lengthens the lag period to exponential growth of <u>Nitrosomonas</u> and thereby inhibits the oxidation of NH_4^+ (McBeath, 1962).

Thiourea has received limited attention as a potential practical means of retarding soil nitrification. McBeath (1962) concluded from laboratory studies that thiourea, added to soil in amounts necessary to exert satisfactory control over soil nitrification, would not adversely affect crops. Confirmational field and greenhouse studies are lacking in this regard, however. Significance must be placed on findings such as those of Fuller (1950) who reported that in the presence of 100 ppm thiourea, rye seedlings suffered leaf tip-burning. Jensen and Bendixen (1958) found that red clover, growing in fields applied with 50 kg/hectare of thiourea, was damaged.

(B) <u>Thioureaformaldehyde Polymers</u>

The ability of a low molecular weight thiourea-formaldehyde polymer to inhibit nitrification was noted previously in pure culture studies. A high molecular weight preparation

was shown to be inactive. In further pursuing these
initial observations, thioureaformaldehyde polymers
 encompassing a wide range of molecular weights were
prepared and assessed by soil incubation methods for in-
hibitory activity. Table 8 summarizes the results of this
study.

The molecular weight of many thioureaformaldehyde
polymers could not be established by vapor phase osmometry.
Insolubility of the synthesized products in dimethyl-
formamide the solvent employed in this technique, was a
common problem among the longer chain polymers. Other
thioureaformaldehyde preparations reacted chemically
with dimethylformamide and proved unsuitable. Nevertheless,
a useful range of molecular weights was established.

Since, in a given polymer preparation, a slight
mixture of longer and shorter chain compounds, in addition
to that of the desired length is realized, the experimen-
tally derived molecular weights may be expected to reflect
this discrepancy. Thus, as anticipated, calculated chain
lengths based upon the experimentally determined molecular
weights and a thioureaformaldehyde monomer molecular weight
of 88, were found to be non-integers.

Table 8: The Inhibition of Soil Nitrification by Chemical Derivatives of Thiourea. Tests Compounds (150 ppm) and Urea (200 ppm) were Applied to 50 gm. of Prepared Soil. (Soil Moisture was Maintained at 2/3 Field Capacity & Incubation was Carried Out at 32°C, Under a Relative Humidity of 98% for 21 Days.)

Chemical Derivative of Thiourea	Experimentally Determined Molecular Weight	Calculated Chain Length	[1] % Inhibition of Nitrate Formation
Thioureaformaldehyde Polymers			
308-28	[2]NR	–	0
27-3	[3]INSOL	–	0
27-2	[4]R_x	–	0
306-5	INSOL	–	0
305-51	INSOL	–	0
307-35	1928	21.9	0
307-36	1570	17.8	4
308-4	803	9.1	8
307-26A	INSOL	–	12
27-1	R_x	–	18
308-7	421	4.8	26
308-2	NR	–	32
307-18	406	4.6	37
118-66-IC	400	4.5	46
MW 30-2	113	1.3	49
307-36 (Recrystallization Product)	R_x	–	49
305-25B	NR	–	54
118-66-16	NR	–	58
307-39B	R_x	–	60
307-25	730	8.3	61
305-14	NR	–	66

Table 8 (Continued)

Condensation Products of Thiourea

$NH_2-\overset{S}{\overset{\|}{C}}-NH-CH_2-NH-\overset{O}{\overset{\|}{C}}-NH-CH_2-NH-\overset{O}{\overset{\|}{C}}-NH_2$ (MW41) 236 21

$CH_3-NH-\overset{S}{\overset{\|}{C}}-NH-CH_2CH_2-NH-C-NH-CH_3$ (P34) 208 23

$NH_2-\overset{S}{\overset{\|}{C}}-NH-NH-\overset{O}{\overset{\|}{C}}-NH_2$ (MW) 134 33

$NH_2-\overset{S}{\overset{\|}{C}}-NH-\overset{OH}{\overset{|}{CH}}CH_2CH_3$ (MW14-1) 137 38

$CH_3-NH-\overset{S}{\overset{\|}{C}}-NH-CH_3$ (P32) 112 42

$NH_2-\overset{S}{\overset{\|}{C}}-NH-CH_3$ (P31) 112 48

$NH_2-\overset{S}{\overset{\|}{C}}-NH-\overset{OH}{\overset{|}{CH}}-CH_2CH_2CH_3$ (MW13-2) 108 50

$\begin{matrix} CH_3 \\ \ \ \ \diagdown \\ \ \ \ \ N-\overset{S}{\overset{\|}{C}}-NH-CH_3 \\ \ \ \ \diagup \\ CH_3 \end{matrix}$ (P33) 116 62

2-Chloro-6-(trichloromethyl)pyridine 94

^{1}% Inhibition if Nitrate Formation $= 100 - \left\{ 100\left(\dfrac{\text{Nitrate concentration in treatment-urea \& inhibitor}}{\text{Nitrate concentration in control-urea}} \right) \right\}$

^{2}No results (NR), molecular weight determinations not carried out

^{3}Insoluble (INSOL) in dimethylsulfoxide, the solvent used in osmometric molecular weight determinations

^{4}Reacts chemically (Rx) with dimethylsulfoxide

Thioureaformaldehyde polymers greater than twenty-two monomeric units in length were largely insoluble in dimethylsulfoxide and exhibited no detectable inhibitory activity. Those thioureaformuldehyde polymers comprised of nine to twenty-two monomeric units were also essentially refractive. Monomers, dimers, trimers and tetramers of thioureaformaldehyde, however, displayed significant ability to inhibit nitrification; activity increasing with decreased chain length. Compounds MW 30-1 and 307-25 posed anomolous results.

Compound 118-66-16 constitutes that fraction of compound 118-66-IC which is soluble in hot water. In light of the correlation between ability to inhibit nitrification and molecular weight of the thioureformaldehyde polymer, the increased inhibitory activity of 118-66-16 over 118-66-IC is not surprising, as one would expect the former to consist of the lighter molecular weight elements of 118-66-IC. A similar rationale may be presented in comparing the activities of 307-36 and 307-36, recrystallized from dimethylsulfoxide.

(C) Condensation Products of Thiourea

The results of soil incubation tests to assess the inhibitory activity of eight substitution products of thiourea are also presented in Table 8. No distinct

relationship between the nature of the chemical substituent
and the resultant ability to inhibit nitrification could be
observed. However, substituted monomers of thiourea
proved noticeably more inhibitory to nitrification than
the long-chain condensation products of thiourea. Sub-
stituted monomers of thiourea and active, low molecular
weight thioureaformaldehyde polymers exhibited similar
inhibitory activities.

(D) 2-Chloro-6-(Trichloromethyl) Pyridine

2-chloro-6-(trichloromethyl) pyridine (N-Serve) is a
semi-commercial nitrification inhibitor developed by
Dow Chemical for agronomic purposes. N-Serve was
included in this study for comparative purposes. In
terms of effective concentration, none of the chemical
derivatives of thiourea approached N-Serve in its ability
to inhibit nitrification (Table 8).

4. An Examination of the Activity of Selected Nitrification
   Inhibitors as a Function of Concentration

In attempts to more fully characterize the ability of
chemical compounds to inhibit nitrification, increasing
inhibitor concentrations graphed against the resultant
percent inhibition of nitrification have proven useful.
Based on quantitative nitrate-nitrogen assays carried out

on inhibitor treated and untreated soil incubation samples,
values for percent inhibition of nitrification were
calculated according to the following expression:

$$\% \text{ inhibition of nitrification} = 100 - \left\{ \left[\frac{\{NO_3^-\} \text{ treatment}}{\{NO_3^-\} \text{ control}} \right] 100 \right\}$$

Inhibitory activity was noted to vary directly with
inhibitor concentration. Plots of inhibitor concentration
vs. percent inhibition of nitrification yielded smooth curves
generally characterized by two distinct slopes. Initially,
percent inhibition of nitrification was observed to vary
rapidly with increasing concentration resulting in
steeply sloped plots. This relationship was found to
hold only through a limited concentration range. An upper
limit to the range of effective inhibitor concentrations
was noted. Application rates surpassing this upper
concentration limit failed to change the percent inhibition
of nitrification appreciably and a state of inhibitor
saturation was observed. Plots in this region decreased
sharply in slope and became asymptotic to the abscissa.

Both the range of effective inhibitor concentrations
and the degree of inhibition achieved prior to inhibitor
saturation were found to be characteristics of the
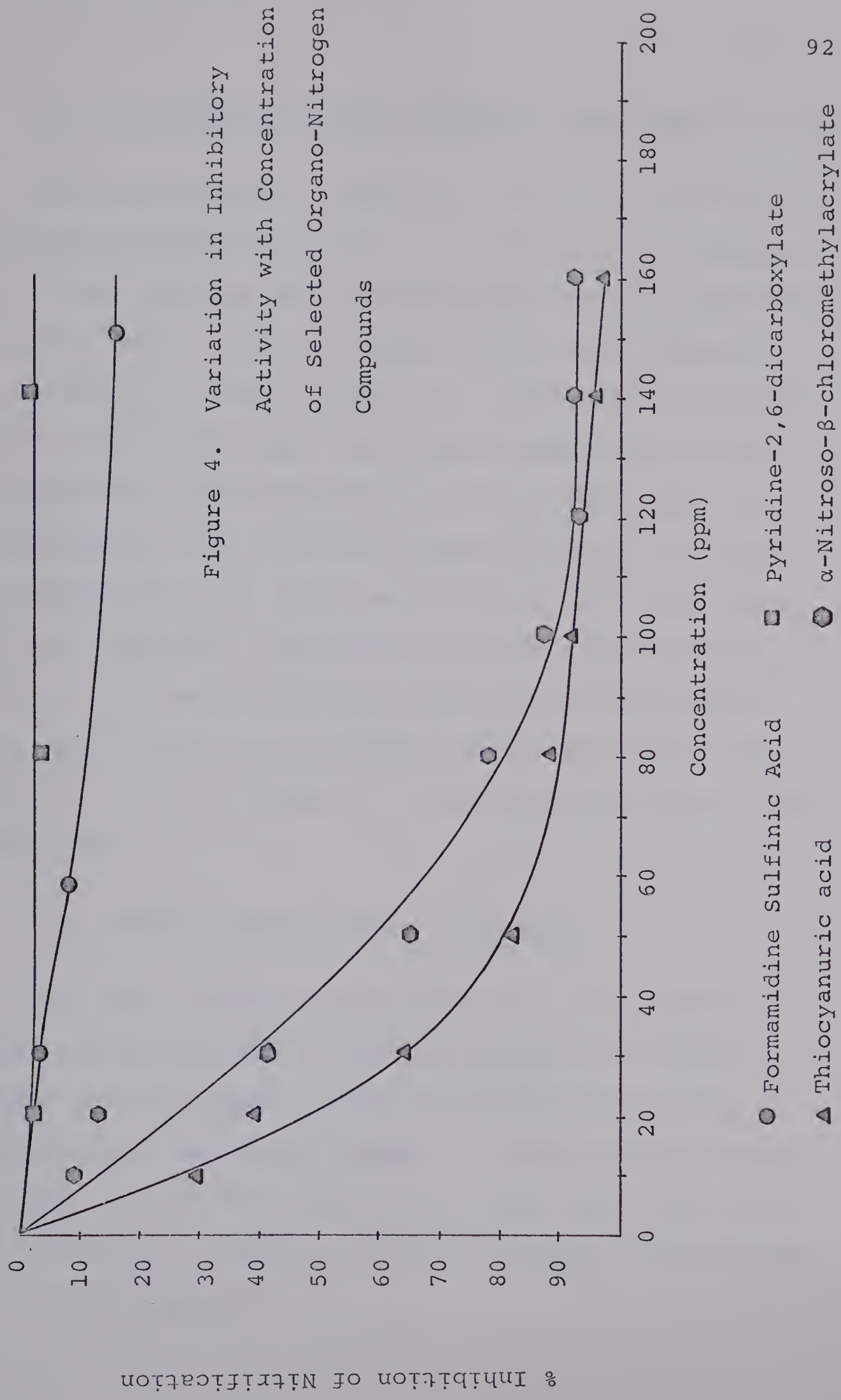chemical under study.

(A) <u>Organo-Nitrogen and Organo-Nitrogen-Sulfur Compounds</u>

The effect upon nitrification of increasing concentrations of three organo-nitrogen compounds (foramidine sulfinic acid, pyridine -2, 6-dicarboxylate, α-nitroso-β-chloromethylacrylate) and a nitrogen and sulfur containing organic compound (thiocyanuric acid) are summarized in Figure 4. In agreement with results presented in section 2 of the results and discussion, the organo-nitrogen compounds, again proved to be significantly inferior to organics possessing both a nitrogen and sulfur grouping i.e thiocyanuric acid, in their ability to inhibit the biological oxidation of urea nitrogen.

As a nitrogen-containing organic, the performance of α-nitroso- β-chloromethylacrylate was anomolous; its inhibitory ability paralleling that of thiocyanuric acid. The effectiveness of this compound as a nitrification inhibitor may perhaps be attributed in part to the presence of a chloride substituent. The practical potential of this chemical is severly limited by the fact that it has been found to be highly phytotoxic in tests with germinating reed canary grass at concentrations of 50 ppm and greater.

Figure 4. Variation in Inhibitory Activity with Concentration of Selected Organo-Nitrogen Compounds

○ Formamidine Sulfinic Acid   ▢ Pyridine-2,6-dicarboxylate   ⬡ α-Nitroso-β-chloromethylacrylate

▲ Thiocyanuric acid

92

(B) <u>Low Molecular Weight Thiourea Formaldehyde Polymers</u>

The performance of 118-66-IC, a low molecular weight thioureaformaldehyde polymer, in inhibiting the formation of nitrate nitrogen, has proven notable, being comparable to highly active organo-nitrogen-sulfur test compounds e.g. thiocyanuric acid (Figure 5). Increasing concentrations of 118-66-IC from 0-100 ppm resulted in a desired corresponding sharp increase in percent inhibition of nitrification; 85% inhibition being achieved at 120 ppm, the concentration at which an inhibitor saturated state is first realized. Phytotoxicity tests conducted with 118-66-IC in conjunction with germinating reed canary grass under controlled greenhouse conditions showed the absence of any ill effects at concentrations even as high as 800 ppm.

(C) <u>Chemical Derivatives of Thiourea</u>

The plots of percent inhibition of nitrification versus concentration of inhibitor presented in Figure 6 display results obtained from an examination of five chemical derivatives of thiourea. The influence of allyl, dimethyl, phenyl and acetyl substituents upon the inhibitory activity of thiourea, while not easily rationalized, were readily observed.
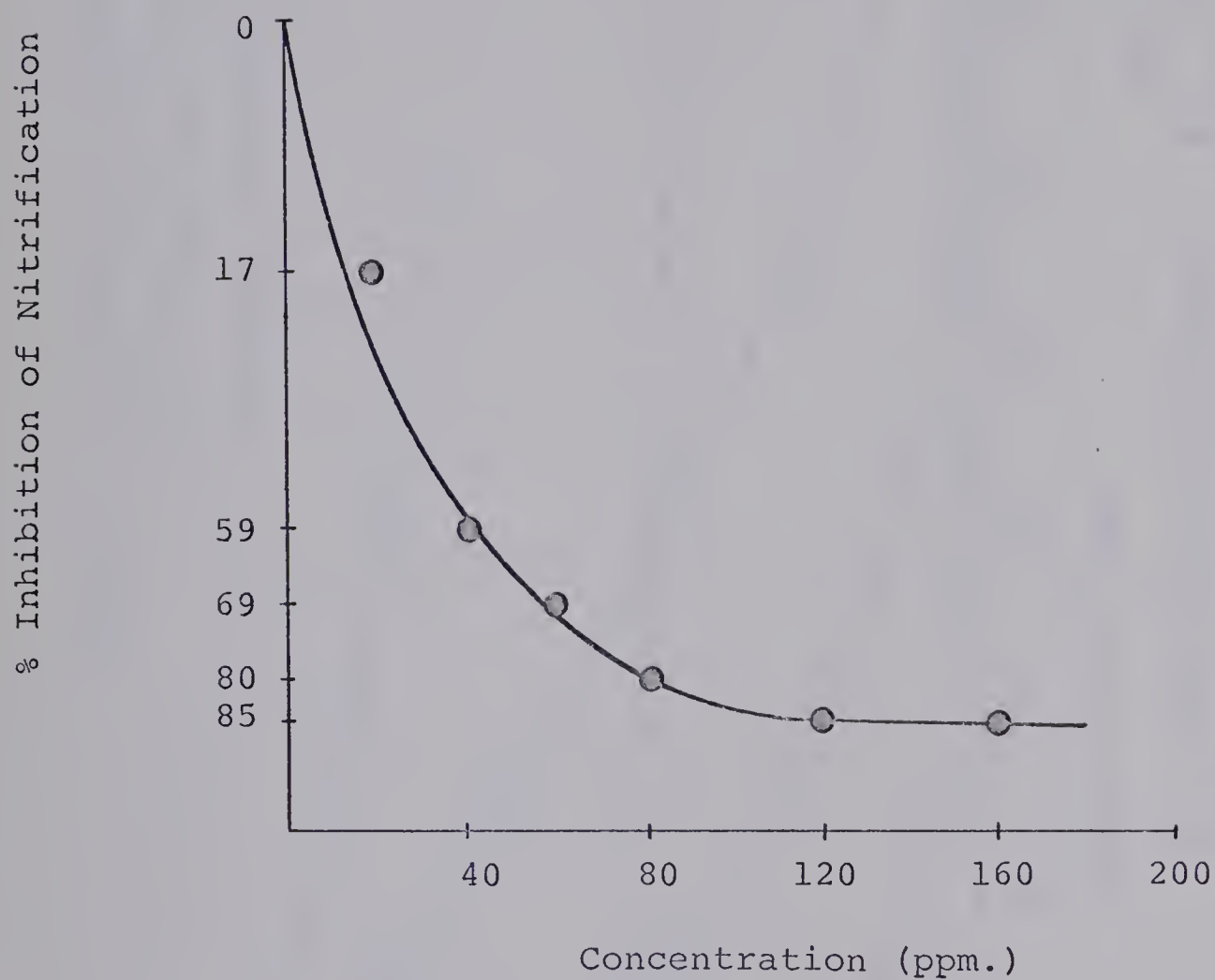
Figure 5. Variation in Inhibitory Activity with
Concentration of 118-66-IC, a Low
Molecular Weight Thiourea-Formaldehyde
Polymer

Figure 6. Variation in Inhibitory Activity
With Concentration of Selected
Thiourea Derivatives

Concentration (ppm.)

% Inhibition of Nitrification

□ N-Acetythiourea   ▨ β-Phenylthiosemicarbazide   ▲ CH_3—N—C—NHCH$_3$ with S double bond

◐ 1-Allyl-2-thiourea   ⬡ 2-Allyl-2-thiopsuedourea hydrochloride

95
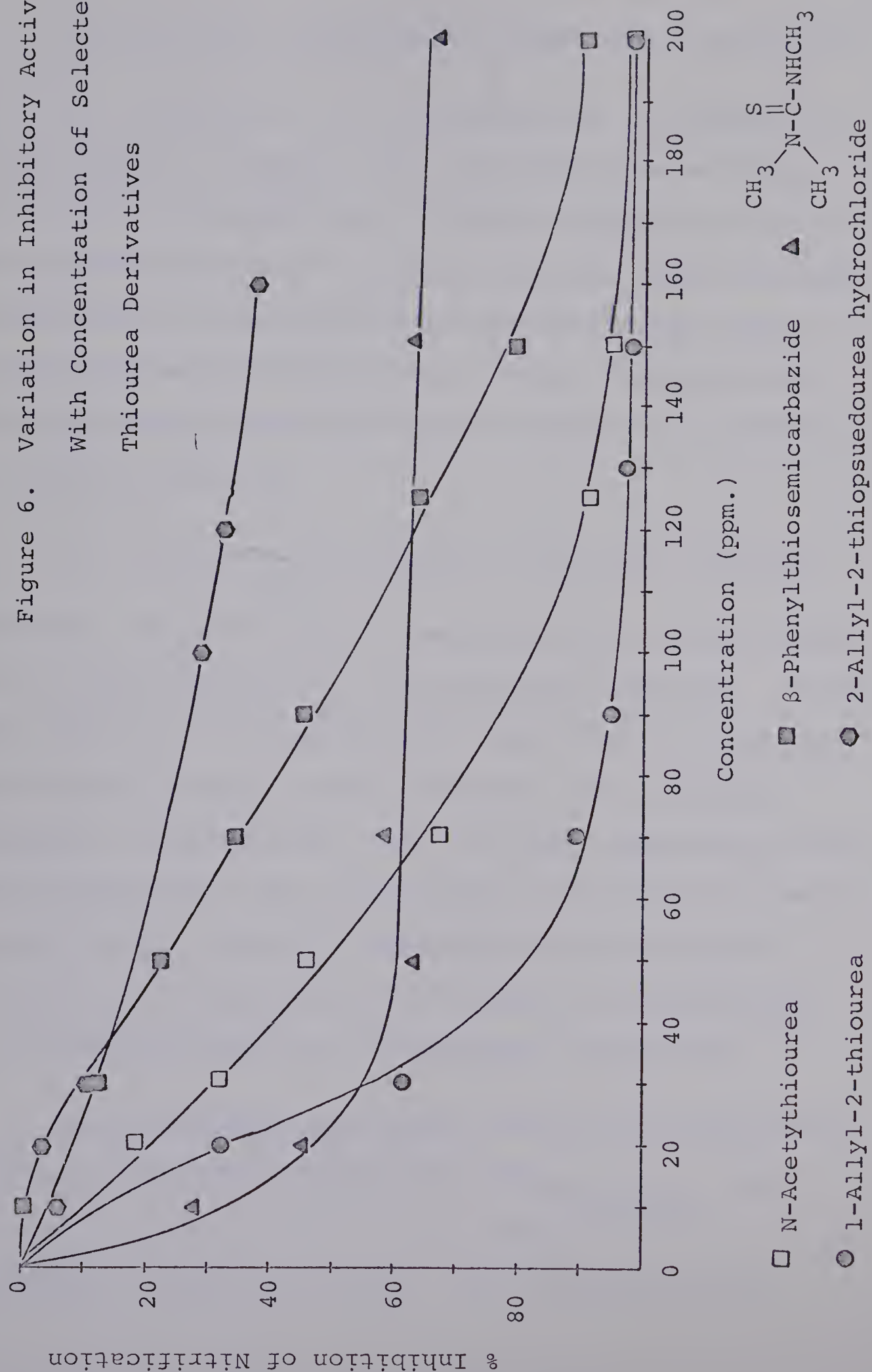
Allylthiourea ($CH_2=CHCH_2NH-C-NH_2$), most notable of
$\overset{\text{||}}{\text{S}}$

the five compounds in its performance as an inhibitor of

nitrification, required only a concentration of 90 ppm

to achieve 95% inhibition of nitrate-nitrogen formation.

The overall activity of allylthiourea was ideal; percent

inhibition of nitrification increasing sharply over a

relatively narrow concentration range. Concentrations

greater than 120 ppm were found to result in an inhibitor

saturated condition.

At concentrations in excess of 150 ppm, N-acetyl-

thiourea ($CH_3-\overset{O}{\overset{\text{||}}{C}}-NH-\overset{S}{\overset{\text{||}}{C}}-NH_2$) closely parallels allylthiourea

in its effectiveness as a nitrification inhibitor. Unlike

the behaviour of allylthiourea, the inhibitory activity

of N-acetylthiourea varies gradually with increasing

chemical concentration. Hence at lower application rates,

N-acetylthiourea lags considerably below allylthiourea in

effectiveness. With 75% inhibition of nitrification

achieved at a concentration of 90 ppm, the performance

of N-acetylthiourea is, nevertheless, significant.

In the concentration range 0-40 ppm, the inhibitory

activity of N,N-dimethylthiourea ($\begin{array}{c} CH_3 \\ \\ CH_3 \end{array}\!\!>\!N-\overset{S}{\overset{\text{||}}{C}}-NH_2$)   was

observed to increase sharply. At a concentration of

40 ppm, 60% inhibitor of nitrification was noted. Applied concentrations greater than 40 ppm resulted in a negligible increase in percent inhibition of nitrification. Only 65% inhibition was recorded at 200 ppm, the highest concentration of N,N-dimethylthiourea tested.

β-phenylthiosemicarbazide ($C_6H_5-NH-\overset{S}{\overset{\|}{C}}-NH_2$) was found to retard nitrification weakly; its ability as an inhibitor was significant only at very high concentration. For β-phenylthiosemicarbazide, percent inhibition of nitrification was observed to vary very gradually, but steadily with increasing chemical concentration, such that at 200 ppm, the highest concentration tested, 90% inhibition was noted.

2-allyl-2-thiopsuedourea hydrochloride ($NH=\overset{\underset{|}{S-CH_2CH=CH_2}}{C}-NH_3^+Cl^-$)
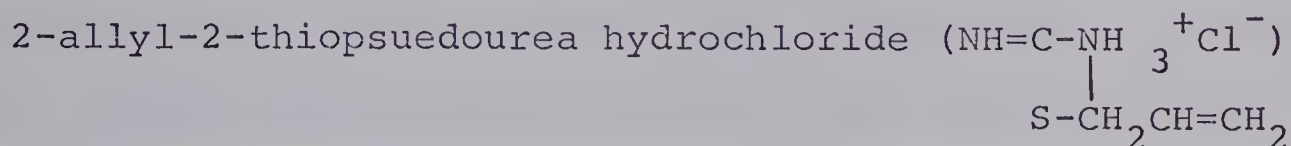
achieved only 35% inhibition at 160 ppm, and was judged to be ineffective as a nitrification inhibitor. Further, growth tests conducted with reed canary grass under greenhouse conditions showed this compound to be phytotoxic at an applied concentration of 50 ppm.

(D) Chlorination Products of 2-Pyridinealdoxime

From references concerning the implications to agriculture of prolonged insecticide and herbicide use,

and from experimental evidence presented in Figure 4 of

section 4 of the results and discussion, the inhibitory

effect of chlorinated hydrocarbons on nitrification has

been clearly established. In this light, the question

of whether or not chlorination can enhance the ability of

an organic compound to inhibit nitrification is of

immediate interest.

In Figure 7, the variation in inhibitory activity with

concentration of three chlorinated derivatives of 2-pyri-

dinealdoxime are examined. 2-pyridinealdoxime itself,

demonstrated a moderate ability to inhibit nitrification;

55% inhibition achieved at a concentration of 100 ppm.

The chlorinated derivatives of 2-pyridinealdoxime; compound

8-69 (singly chlorinated derivative) and compounds 7-69

and 8-69 (hydrochloride salts of the singly chlorinated

derivative), did not display an appreciable increase in

inhibitory activity over the parent compound. In fact,

compound 8-69 was less active than 2-pyridinealdoxime.

Compound 7-69, with two functional chloride groupings,

appeared to be slightly more effective an inhibitor than

either the parent compound or the singly chlorinated

derivative at higher concentrations. Compound 9-69, while

similar in composition but not in structure to 7-69,

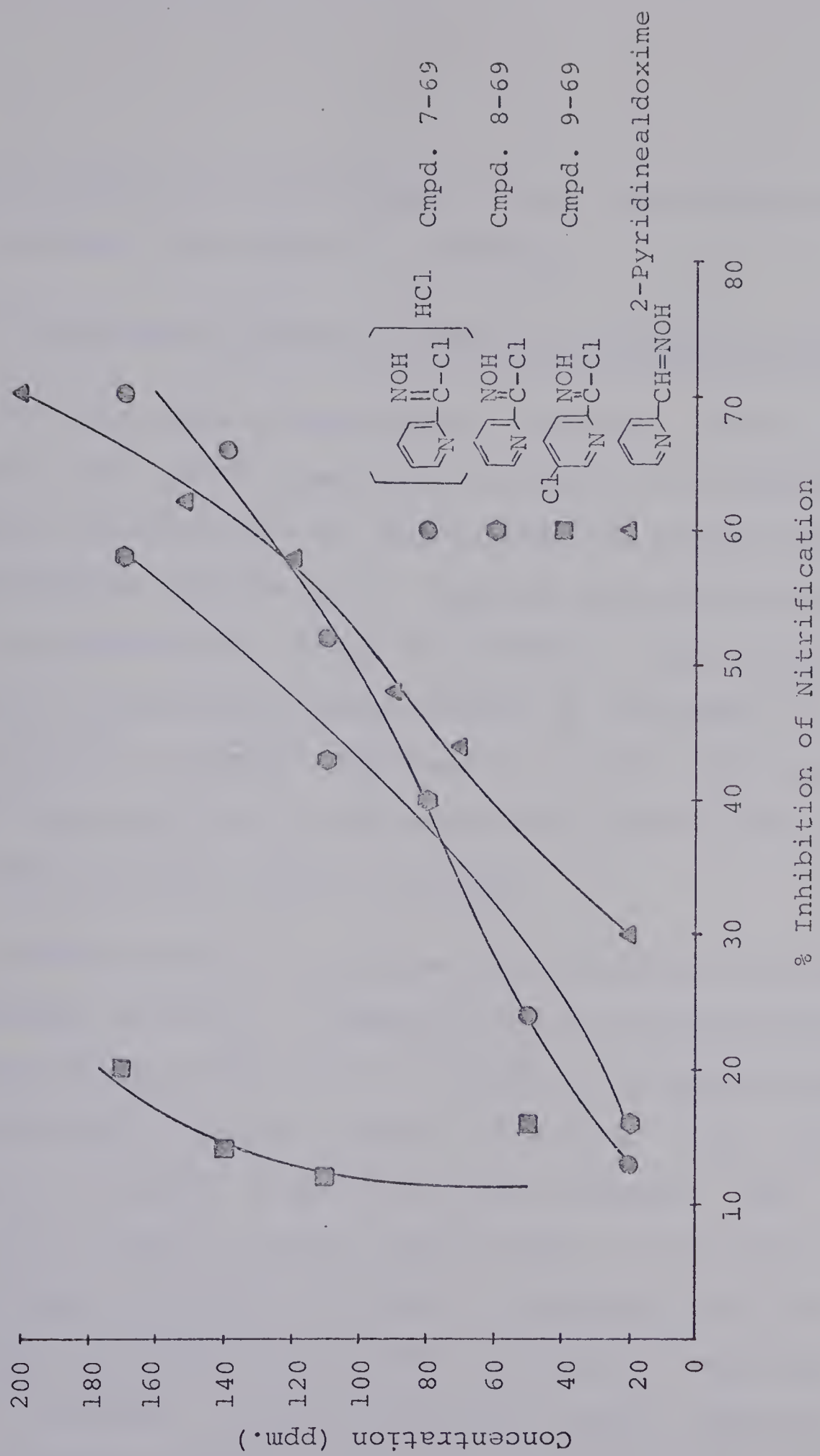exhibited a dramatic loss in inhibitory activity and

Figure 7: Variation in Inhibitory Activity with Concentration of Selected
Chlorinated Derivatives of 2-Pyridinealdoxime.

would suggest that positioning of the chloride molecule
is critical with respect to activity.

5. High Molecular Weight Thioureaformaldehyde Polymers

In the light of experiments conducted earlier in this
study, which established that the ability to effectively
inhibit nitrification was associated with those thiourea-
formaldehyde polymers less than 800 in molecular weight,
it is reasonable to assume that should a high molecular
weight thioureaformaldehyde polymer be suceptible to
biological or chemical degradation in soil, detectable
inhibitory activity should arise with time and the formation
of lower molecular weight entities.

The influence of prolonged soil incubation upon the
inhibitory activity of compound 27-3, a thioureaformal-
dehyde polymer found to be insoluble in dimethyformamide
and therefore judged to possess a molecular weight well in
excess of several thousand, has been examined. The
results of table 9, would indicate that on the basis of
inhibitory activity, virtually no degradation of the
polymer has occurred even after 156 days of continual
soil incubation. The high molecular weight thiourea-
formaldehyde polymers would appear to be highly refractive
and stable.

Table 9: Variation in the Inhibitory Activity of a High Molecular Weight Thiourea-Formaldehyde Polymer (27-3) with Time Under Continual Incubation. Incubation was Carried Out at 23°C, Under Ambient Relative Humidity, in Soil Maintained at 2/3 Field Capacity.

Accumulated Period of Incubation (Days)	Nitrate-Nitrogen Concentration (μg/gm. Soil)		% Inhibition of Nitrification
	Control	27-3	
21	442	440	0.4
40	420	422	—
63	420	418	0.9
94	415	415	—
118	413	410	0.7
156	405	400	1.2

6. <u>Inhibitor Coating of Urea as a Method of Nitrogen</u>

   <u>Conservation</u>

Table 10 summarizes the results of a study conducted to assess the effectiveness of inhibitor coating as a means of controlling the transformation of urea nitrogen to nitrate nitrogen in soil. Coated urea granules were prepared and provided through the courtesy of the Research Council of Alberta. Product specifications appear in appendix 7.

That the conversion of inhibitor coated urea to nitrate was being retarded in comparison to that for untreated urea was clearly evident after two weeks. The degree of control exercised over nitrate-nitrogen formation was found to vary, as expected, with the chemical chosen as a coating.

The performance of 2-chloro-6-(trichloromethyl)-pyridine (N-serve) was most notable; 51% inhibition of nitrification being achieved at an applied inhibitor concentration of only 1 ppm. Such results, however, were well below the expected capabilities of N-serve. According to Goring (1962a), 75% control of nitrification was obtained for twelve weeks with an applied concentration of N-serve of 1 ppm.

Table 10: Control of Nitrate-Nitrogen Formation Employing

Inhibitor-Coated Urea Granules. Application

Rates: Nitrogen as Urea: 200 ppm, Low Molecular

Weight Thiourea-Formaldehyde Polymers (Compounds

118-66-IC, 305-14, 305-258) - 22 ppm, 2-Pyri-

dinealdoxime: 22 ppm, N-Serve: 1 ppm. (Incu-

bation was carried out at 32°C, under 98%

relative humidity, in soil maintained at 2/3

field capacity).

Treatment	*N Recovered as NO_3-N	
	2 wks	4 wks
Urea	100	100
Urea & 118-66-IC	74	71
Urea & 305-14	66	90
Urea & 305-25B	80	102
Urea & 2-Pyridine-Aldoxime	46	61
Urea & N-Serve	49	82

*Quantitative nitrate-nitrogen values presented have been

compared to that of the control, the latter arbitrarily

equated to a value of 100.

The thioureaformaldehyde polymers proved least effective
of the chemicals chosen as inhibitor coatings. Representa-
tive of this group of compounds is 118-66-IC, which at
an applied concentration of 22 ppm retarded nitrate nitrogen
formation by only 26%. These results, however, agree
well with previous findings in which 118-66-IC was applied
separately (Section 4, Figure 5). It would appear that
the intimate positioning of the inhibitor with respect to
the nitrogen source, insured by coating, does not prove
significantly advantageous.

With the exception of compound 118-66-IC, which
maintained its former level of control over nitrate nitrogen
formation all inhibitors showed a marked loss in
ability to retard the oxidation of urea-nitrogen after
four weeks of continual incubation. Particularily notice-
able was the loss in activity of 2-chloro-trichloro-
methylpyridine a fact in keeping with the literature.

Loss of N-serve has been attributed to two exponential
processes; volatilization and degradation to 6-chloropicolo-
nic acid (Redemann, Meikle and Widofsky, 1964). Enhanced
by low soil pH and low soil organic matter, the loss
of N-serve, reported in terms of half-life, may be as short
as four days at 20°C.

Although experiencing a considerable loss in
inhibitory activity during the 3rd and 4th weeks of
incubation in soil, 2-pyridinealdoxime was nevertheless
observed to exercise the best control over nitrate-nitrogen
formation (39% inhibition).

7. 118-66-IC, a Low Molecular Weight Thiourea-Formaldehyde
    Polymer

    (A) An Examination of Nitrogen Sources and Their
        Influence Upon the Inhibitory Activity of
        118-66-IC

That 2-chloro-6-(trichloromethyl) pyridine (N-Serve)
specifically inhibits the ammonium and not the hydroxy-
lamine oxidase system of Nitrosomonas and leaves
unaffected the nitrite cytochrome oxidase and reductase
systems in Nitrobacter has been established by Campbell
and Aleem (1965, I,II) and Goring (1962 a,b). In agreement
with such findings are the results of Table II, where N-Serve,
at a rate of 100 ppm., was found to inhibit essentially
completely (98% - 99%) the oxidation of urea and ammonium
nitrogen. As expected, inhibition of nitrite nitrogen with
N-Serve was markedly reduced (69%).

Essentially complete inhibition (94% - 98%) of nitrate
formation from urea and ammonium was also noted with an

Table 11: Effect of Nitrogen Sources Upon the Ability of 118-66-IC (A Low-Molecular Weight Thiourea-Formaldehyde Polymer) and 2-Chloro-6-Trichloro-methylpyridine (N-Serve); to Inhibit the Formation of Nitrate-Nitrogen in Soil. Application Rate of the Nitrogen Sources was 100 ppm; of 118-66-IC, 400 ppm and of N-Serve, 100 ppm. Incubation in Soil Held at 2/3 Field Capacity, was at 32°C Under a Relative Humidity of 98% for 21 Days.

Nitrogen Source	Inhibitor	% Inhibition of NO_3-N Formation
Urea	118-66-IC	97
Urea	N-Serve	98
NH_4^+	118-66-IC	97
NH_4^+	N-Serve	94
NO_2^-	118-66-IC	86
NO_2^-	N-Serve	69

application of 400 ppm 118-66-IC. A slight but perhaps
significant, loss in ability to inhibit nitrite oxidation
was observed for 118-66-IC. If it is hypothesized that
the degradation of 118-66-IC yields free thiourea molecules,
inability to prevent nitrite oxidation may be expected since
thiourea has been reported as a specific inhibitor of
Nitrosomonas (Alexander, 1965). That free thiourea
constitutes the sole source of inhibitory activity in
118-66-IC is questionable, however, since a high degree of
inhibition of nitrate formation (86%) was noted even with
a nitrite nitrogen source.

(B) The Response of Greenhouse Grown Reed Canary
     Grass to Varying Concentrations of 118-66-IC/urea

Yields of Reed Canary grass grown under varying
concentrations of 118-66-IC and urea compared to that
obtained from control treatments in which 200 ppm nitrogen
was supplied solely as urea. The results of this study
appear in figure 8.

Examination of the results for the first harvest, taken
after four weeks growth, showed that for those treatments
receiving even as little as 10 ppm 118-66-IC [1] (concentration

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[1] 118-66-IC has a nitrogen content of 25.3%. Therefore
conversion of concentration expressed on the basis of
nitrogen content of 118-66-IC to concentration expressed
on the basis of the compound as a whole requires multiplica-
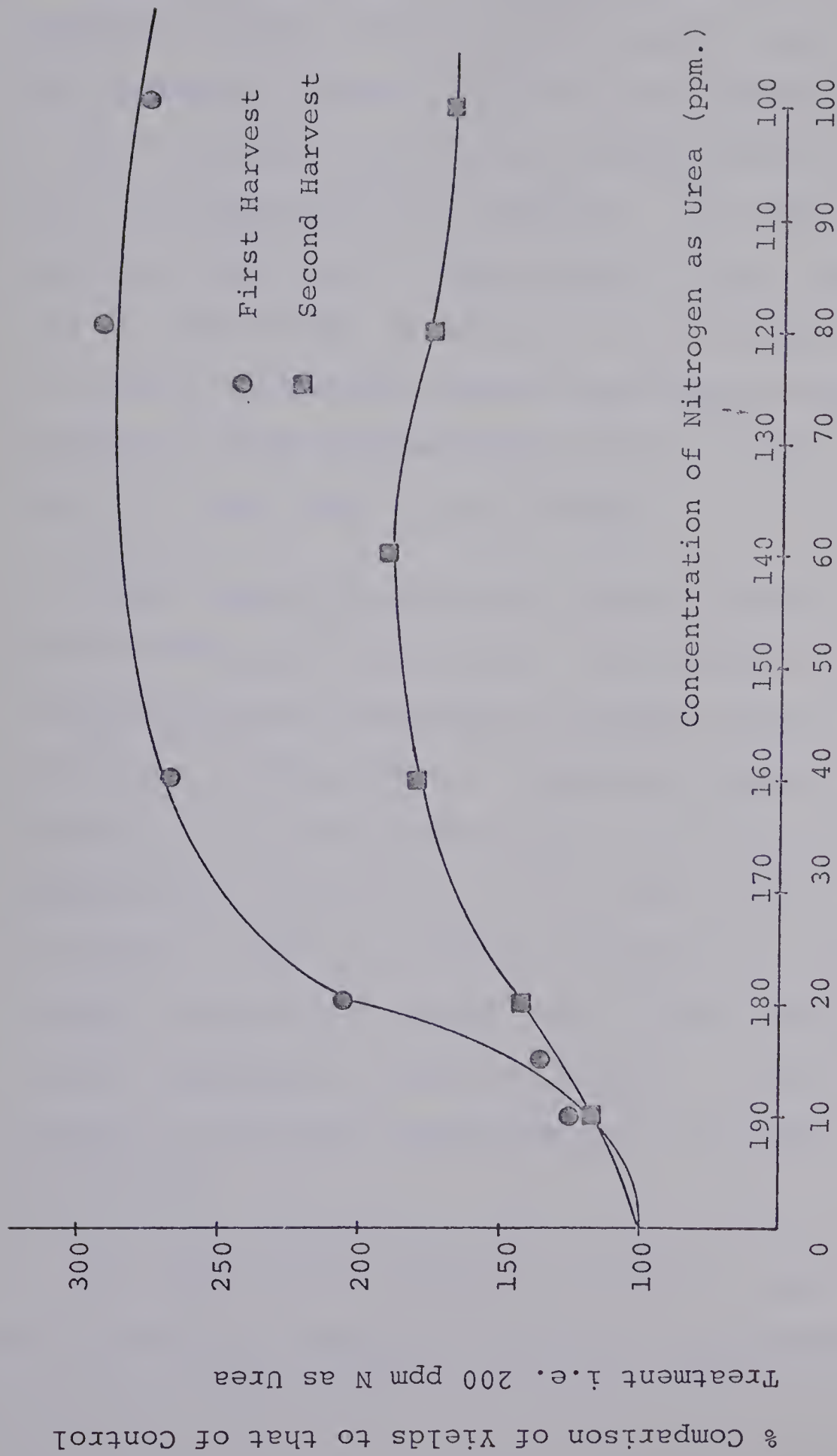tion of the former by a factor of 3.95

Figure 8. Effect Upon Greenhouse Grown Reed Canary Grass Yields by
Variations in Urea/118-66-IC Application Rates

expressed on the basis of nitrogen content), a significant increase in grass yields over the control was realized. This increase in grass yields over the control was found to vary inversely with the urea concentration and directly with the concentration of 118-66-IC. For those treatments receiving less than 170 ppm nitrogen as urea and greater than 30 ppm nitrogen as 118-66-IC i.e. 118 ppm inhibitor as a whole, a levelling effect was noted in which treatment yields remained essentially constant at approximately 1.75 times that of the control.

Eight weeks following the initial harvest, a second cutting was carried out. As evident in figure 8, treatment yields consistently surpassed those of the control to a marked degree. Treatment yields again varied inversely with urea concentration and directly with the concentration of 118-66-IC. As in the initial harvest, the yields of those treatments receiving less than 170 ppm nitrogen as urea and greater than 118 ppm 118-66-IC as a whole, displayed a levelling effect. In this region treatment yields were approximately 2.75 times that of the control.

In comparing the results of figure 5 (pp. 94), in which the inhibitory activity of 118-66-IC was examined as a

function of concentration, and figure 8, good correlation

of data was observed. For 118-66-IC, an inhibitor

saturated condition i.e. a state of maximum inhibitory

activity, was observed at 120 ppm (figure 5). Correspondingly,

peak yields were associated with those treatments receiving

greater than 118 ppm of 118-66-IC as a whole (figure 8).

(C) <u>Time as a Factor in the Inactivation of 118-66-IC</u>

<u>in a Soil Environment</u>

That 118-66-IC retains its activity as a nitrification

inhibitor in soil for an extended period of time is

evident from the data presented in tables 12 and 13.

Even after thirteen months of continuous incubation

under greenhouse conditions, both, soil previously applied

with 118-66-IC and 118-66-IC coated onto diatomite,

demonstrated unquestionable activity in retarding nitrate

nitrogen formation (table 12). In comparing the results

of figure 5 (pp. 94), in which the inhibitory activity

of 118-66-IC was examined as a function of concentration

and table 12, some loss in activity was noted with

prolonged soil incubation. This fact was especially apparent

with the lower applied rates of inhibitor.

Soil samples obtained from field plots initially

applied with 1000 lb/acre of 118-66-IC and maintained for

Table 12: An Assessment of the Residual Inhibitory Activity

of Compound 118-66-IC, a Low Molecular Weight

Thiourea-Formaldehyde Polymer, After Thirteen

Months of Continual Incubation in Soil Under

Standard Greenhouse Conditions (23°C, 2/3 Field

Capacity, Ambient Relative Humidity). Urea N

at 200 ppm was Supplied as the Nitrogen Source.

Inhibitor	Inhibitor Conc. (ppm.)	Nitrate Nitrogen Formed (μg/gm Soil)	% Inhibition of NO_3-N Formation
118-66-IC	0	61	0
118-66-IC	80	84	—
118-66-IC	160	38	36
118-66-IC	240	26	58
118-66-IC	320	22	64
118-66-IC	400	9	85
118-66-IC	800	5	92
118-66-IC on Diatomite	80	88	—
118-66-IC on Diatomite	160	42	31
118-66-IC on Diatomite	240	37	39
118-66-IC on Diatomite	320	6	89
118-66-IC on Diatomite	400	3	95
118-66-IC on Diatomite	800	2	96

30 months, exhibited significant inhibitory activity;
53% inhibition of nitrate nitrogen formation being observed
in soil incubation tests (Table 13). The definite but
limited inactivation of 118-66-IC with time and exposure
to a soil environment was again, however, evident.

Table 13.   An Assessment of the Residual Inhibitory Activity

of 118-66-IC, a Low Molecular Weight Thiourea-

formaldehyde Polymer, after 30 Months of

Continual Incubation under Field Conditions.

Inhibitor was Surface Applied as a Water-

Slurry at a Rate of 1000 lb/Acre to Test Plots

Located on Malmo Clay Loam Seeded to Pasture

Grasses

Treatment Description	Concentration of NO$_3$-N Formed (g/gm Soil)	% Inhibition of NO$_3$-N Formation
Control Soil & Urea	58	0
Control Soil - Unammended	27	—
Thiourea-Formaldehyde Treated Soil & Urea	27	53%
Thiourea-Formaldehyde Treated Soil- Unammended	27	—

V. SUMMARY AND CONCLUSIONS

Seventy chemicals; twenty-three sulfur and nitrogen containing organics, eighteen organo-nitrogen compounds and twenty-nine polymeric preparations including those of thiourea and formaldehyde were assessed for agricultural potential as inhibitors of nitrate formation in soil. The following conclusions have been drawn.

1. Minor modifications designed to hasten analysis can be made to the standard phenoldisulfonic acid method for quantitative nitrate nitrogen determination (Bremner, 1965) with virtually no sacrifice to accuracy.

2. The use of purified cultures of autotrophic nitrifying bacteria is inadequate as a means of reliably screening test compounds for potential as nitrification inhibitors. Chemical concentrations 10-80 times that used in studies with purified cultures are required to produce comparable levels of inhibition in soil incubation tests.

3. A correlation exists between inhibitory activity and potential ability to chelate divalent metal ions strongly.

4. With the exception of 1,2 diamino-N,N'-bis(methyl-2-hydroxy-5-methylbenzyl) ethane and bis(salicyaldehyde) ethylenediimine, the organo-nitrogen compounds prove inferior to the organo-nitrogen and sulfur-containing compounds as inhibitors of nitrification. Among the nitrogen

and sulfur-containing organics, the inhibitory activity
of thiocyanuric acid and 2-thiouracil is notable.

5.   Organo-nitrogen and sulfur-containing compounds with
both the nitrogen and sulfur atoms on the same carbon
atom prove to be particularily strong nitrification inhi-
bitors. Allylthiourea, N-acetylthiourea, N'N(dimethyl)-N'-
(methyl) thiourea and the low molecular weight thiourea-
formaldehyde polymers are noteable examples in this respect.

6.   2-pyridinealdoxime, an organo-nitrogen compound,
inhibits nitrification by 53% @ 100 ppm. Chlorination of
this compound does little to increase the inhibitory ability
of the parent compound.

7.   Of the polymers of thiourea and formaldehyde, only those
less than 800 in molecular weight display significant
ability to inhibit nitrification. Their inhibitory activity
increases with decreasing molecular weight.

8.   Effective control of nitrate formation from urea can be
obtained by coating granules of the latter with suitable
nitrification inhibitors.

9.   None of the chemical compounds tested prove to be as
effective as 2-chloro-6-(trichloromethyl) pyridine in
inhibiting nitrification. Inactivation of N-serve in soil
is surprisingly rapid; significant loss in activity noted
after only three weeks.

10. Compound 118-66-IC, a polymer of thiourea and
formaldehyde having a molecular weight of 400, displays
promising activity; 80% - 85% inhibition of nitrification
being observed with an application rate of 120 ppm. 118-
66-IC proves to be non-phytotoxic even at concentrations
as high as 800 ppm. and, in greenhouse tests, significantly
increases grass yields through nitrogen conservation. As
a nitrification inhibitor, 118-66-IC remains active in
soil for periods exceeding thirteen months in greenhouse
tests and thirty months under field conditions. 118-66-IC
retards nitrate formation from urea and ammonium nitrogen
sources slightly more effectively than from a nitrite
nitrogen source.

BIBLIOGRAPHY

Akundov, F.G. 1968. The relation between leaching of nutrient elements from soil and the use of ferti-lizers. Abstr. Soils and Fert. 31: 1965.

Aleem, M.I.H. and M. Alexander. 1958. Cell-free nitrifi-cation by Nitrobacter. J. Bacteriol. 76:510-514.

_____, M.S. Engle and M. Alexander. 1957. Inhibition of nitrification by ammonia. Bacteriol. Proc. 57:9.

_____, and H. Lees. 1963. Autorophic Enzyme Systems 1. Electron transport systems concerned with hydroxy-lamine oxidation in Nitrosomonas. Can. J. Biochem. and Physiol. 41: 763-778.

_____, H. Lees, R. Lyric and D. Weiss. 1962. Nitrohydroxy-lamine - the unkown intermediate in nitrification? Biochem. Biophys. Research Comm. 7: 126-127.

_____, and A. Nason. 1959. Nitrite oxidase, a particulate cytochrome electron transport system from Nitro-bacter. Biochem. Biophys. Research Comm. 1: 323-327.

_____, and A. Nason. 1960. Phosphorylation coupled to nitrite oxidation by particles from the chemautotroph Nitrobacter agilis. Proc. Natl. Acad. Sci. U.S. 46: 763-769.

Alexander, M. 1965. Nitrification In Soil Nitrogen, W.V. Bartholomew and F.E. Clark (ed.). A.S.A. monograph 10. Am. Soc. Agron., Madison, Wisconsin.

_____. 1967. Introduction to Soil Microbiology. John Wiley and Sons, Inc. New York.

Allison, F.E. 1955. The enigma of soil nitrogen balance sheets. Adv. in Agron. 7: 213-217.

_____. 1963. Losses of gaseous nitrogen from soils by chemical mechanisms involving nitrous acid and nitrites. Soil Sci. 96: 404-409.

_____, W.B. Bollen and C.D. Moodie. 1965. Total Carbon In Methods of Soil Analysis, Part II, Chemical and Microbiological Properties, C.A. Black (Ed. in chief). A.S.A. monograph 9. Am. Soc. Agron., Madison, Wisconsin.

_____, J.H. Doetsch and L.D. Sterling. 1952. Nitrogen gas production by interaction of nitrites and amino acids. Soil Sci. 74: 311-314.

Anderson, J.H. 1959. Studies on the biochemistry of ammonia oxidation by the autotrophic nitrifying organism Nitrosomonas. Ph.D. thesis, Aberdeen, Scotland (Quoted in Lees, 1963).

Andreae, W.A. 1963. Herbicides. In Metabolic Inhibitors, Vol. II. R.M. Hochster and J.H. Quastel (ed.) pp. 243-258. Academic Press, New York.

Arnon, D.I. 1937. Ammonium and nitrate nitrogen nutrition of barley at different seasons in relation to hydrogen ion concentration, magnesium, copper and oxygen supply. Soil Sci. 44: 91-121.

Association of Official Agricultural Chemists. 1955. Official Methods of Analysis, 8th edition. Washington 4, D.C.

Atanasiu, N., A. Westphal, and A.K. Banerjee. 1968. Studies on the effect of nitrogen fertilizers on yield and nitrogen uptake of plants in soils of different ammonium fixing capacities. Abstr. Soils and Fert. 31: 2766.

Baker, A.S. 1967. Colorimetric determination of nitrate in soil and plant extracts with brucine. J. Agric. Fd. Chem. 15: 802-806.

Bates, T.E. and S.L. Tisdale. 1959. Movement of nitrate nitrogen through columns of coarse-textured soil materials. Soil Sci. Am. Proc. 21: 525-528.

Beaton, J.D., W.A. Hubbard and R.C. Speer. 1967. Coated urea, thiourea, urea-formaldehyde, hexamine, oxamide, glycouril and oxidized nitrogen-enriched coal as slowly available sources of nitrogen for Orchardgrass. Agron. J. 59: 127-133.

Bizzell, J.A. 1944. Lysimeter experiments, VI. The effects
of cropping and fertilization on the loss of
nitrogen from the soil. Cornell Agr. Exp. Sta.
Memo 256: 1-14. (Quoted in Allison, 1965).

Bremner, J.M. 1956. Inorganic forms of nitrogen. In
Methods of Soil Analysis, Part II, Chemical and
Microbiological Properties. C.A. Black (ed. in
chief) A.S.A. monograph 9. Am. Soc. Agron.,
Madison, Wisconsin.

_____. and K. Shaw. 1958. Denitrification in soil I.
Methods of investigation. J. Agric. Sci. 51: 22-39.

_____. and K. Shaw. 1958. Denitrification in soil II.
Factors affecting denitrification. J. Agric. Sci.
51: 40-51.

Broadbent, F.E. and F.E. Clark. 1965. Denitrification.
In Soil Nitrogen, W.V. Bartholomew and F.E. Clark
(ed.) A.S.A. monograph 10. Am. Soc. Agron.,
Madison, Wisonsin.

_____. and T. Nakashima. 1968. Plant uptake and residual
value of six tagged nitrogen fertilizers. Soil
Sci. Am. Proc. 32: 388-392.

Brown, M.J., R.E. Luebs and P.F. Pratt. 1966. Effect of
temperature and coating thickness on the release of
urea from resin-coated granules. Agron. J. 58: 175-
178.

Brown, W.T., J.H. Quastel and P.G. Scholefield. 1954.
Effect of mercapto compounds on soil nitrification.
Appl. Microbiol. 2: 235-234.

Broyer, T.C. 1951. The nature of the process of inorganic
salt accumulation in roots. In Mineral Nutrition
of Plants, E. Truog (ed.) Univ. of Wis. Press,
Madison, Wisconsin (Quoted in Viets, 1965).

Buckett, J. 1955. Nitrate determination via 2,4-xylenol
method. Analyst 80: 141.

Butts, W.D. 1960(a). Nitrite oxidation by Nitrobacter
in the presence of certain nitrophenols. Nature 188:
147-148.

_____. 1960(b). The biochemistry of the nitrifying organisms, VI. The effect of oxygen concentration on nitrite oxidation in the presence of different inorganic ions. Biochem. J. 76: 425-427.

Cady, F.B. and W.V. Bartholomew. 1960. Sequential products of anaerobic denitrification in Norfolk soil material. Soil Sci. Am. Proc. 24: 477-482.

Campbell, N.E.R. and M.I.H. Aleem. 1965. The effect of 2-chloro-6-(trichloromethyl) pyridine on the chemautotrophic metabolism of nitrifying bacteria. I. Ammonia and hydroxylamine oxidation by Nitrosomonas. Antonie van Leeuwenhoek 31: 124-136.

_____. and M.I.H. Aleem. 1965. The effect of 2-chloro-6-(trichloromethyl) pyridine on the chemautotrophic metabolism of nitrifying bacteria. II. Nitrite oxidation by Nitrobacter. Antonie van Leeuwenhoek 31: 137-144.

Caseley, J.C. and F.E. Broadbent. 1968. The effect of 5 fungicides on soil respiration and some nitrogen transformations in Yolo fine sandy loam. Abstr. Soils and Fert. 31: 3246.

Cawse, P.A. 1967. The determination of nitrate in soil solutions by U.V. spectroscopy. Analyst 92: 311-315.

Chapman, H.D., G.F. Liebig and D.S. Rayner. 1949. A lysimeter investigation of nitrogen gains and losses under various systems of covercropping and fertilization and a discussion of error sources. Hilgardia 19: 57-128.

Clark, F.E. 1962. Losses of nitrogen accompanying nitrification. International Soil Conference Transactions, New Zealand, 1962. pp. 173-176.

Clarke, A.L. and A.C. Jennings. 1965. Spectrophotometric estimation of nitrates in soil using chromotropic aid. J. Agric. Fd. Chem. 13: 174-176.

Cooper, G.S. and R.L. Smith. 1963. Sequence of products formed during denitrification in some diverse Western soils. Soil Sci. Am. Proc. 27: 659-662.

Curtis, O.F. and D.G. Clark. 1950. An Introduction to
      Plant Physiology. McGraw-Hill Book Co. Inc.,
      New York.

Delwiche, C.C. 1961. Reaction of cell-free preparations
      of Nitrosomonas europaea Abstr. Fed. Proc. 20: 350.

DeWit, C.T. 1963. Ionic balance of the Growth of Plants.
      Versl. Landboowk. Onderz. 69: 15 (quoted in Viets,
      1965).

Dominion Bureau of Statistics (Manufacturing and Primary
      Industries Division) 1968. Fertilizer Trade.

Doughty, J.L. 1941. The advantages of soil paste for
      routine pH determinations. Sci. Agr. 22: 135-138.

Downing, A.L., T.G. Tomlinson and G.A. Truesdale. 1964.
      Effect of Inhibitors on Nitrification in the
      Activated-sludge Process. J. Proc. Inst. Sewage.
      Purification, Part 6. pp. 537.

Easthoe, J.G. and A.G. Pollard. 1950. Nitrate determination
      via the phenoldisulphonic acid method. J. Sci.
      Fd. Agric. 1: 266.

Eno, C.F. 1968. Effects of soil applications of ten
      chlorinated hydrocarbon insecticides on soil micro-
      organisms and the growth of stringless Valentine
      beans. Soil Sci. Am. Proc. 22: 235-238.

Elylar, O.R. Jr., E.L. Schmidt. 1959. A survey of
      heterotrophic micro-organisms from soil for the
      ability to form nitrite and nitrate. J. Gen.
      Microbiol. 20: 473-481.

Fischer, F.L. 1958. Inorganic nitrate, nitrite or nitrate-
      nitrite: rapid colorimetric determinations of
      microgram quantities in aqueous solution. Analytical
      Chem. 30: 1972-1974.

Fuller, W.H. 1950. Behaviour of nitrogenous fertilizers in
      alkaline calcareous soils: 1. Nitrifying character-
      istics of some organic compounds under controlled
      conditions. Ariz. Agri. Exp. Sta. Tech. Bull. 120:
      451-467.

Gardner, W.R. 1958. Laboratory studies of evaporation from soil columns in the presence of a water table. Soil Sci. 85: 244-249.

Gasser, J.K. 1964. Inhibiting the nitrification of ammonium salts. Rep. Rothamstead Exp. Sta. 1963: 46.

_____. 1965. N-serve. Abstr. Soils and Fert. 28: 3880.

_____. 1967. Measurement of losses of fertilizer nitrogen during incubation in acid sandy soils and during subsequent growth of ryegrass using N^{15}-labelled nitrogen. J. Soil Sci. 18: 289-300.

_____. 1968. The effects on winter wheat of ammonium sulfate; with and without a nitrification inhibitor, and of calcium nitrate. Abstr. Soils and Fertilizer 32: 715.

Gayon, U., G. Dapeit. 1886. Recherches sur la reduction des nitrates par les infiniments petits. Soc. Sci. Phys. Nat. Bordeaux. Ser. 3: 201-307. (Quoted in Broadbent, 1965).

Gerretson, A.L. 1968. Inhibition of nitrifying chemolitho-trophic bacteria by several insecticides. J. Econ. Ent. 61: 285-288.

Gerretson, F.C. and H. de Hoop. 1959. Nitrogen losses during nitrification in solutions and in acid sandy soils. Can. J. Microbiol. 3: 359-380.

Goring, C.A.I. 1962a. Control of nitrification by 2-chloro-6-(trichloromethyl)pyridine. Soil Sci. 93: 211-218.

_____. 1962b. Control of nitrification of ammonium fertilizers and urea by 2-chloro-6-(trichloromethyl) pyridine. Soil Sci. 93: 431-439.

Hageman, R.H. and D. Flesher. 1960. Nitrate reductase activity in corn seedlings as affected by light and nitrate content of nutrient media. Plant Plysiol. 35: 700-708.

Hale, M.G. 1957. The effects of several herbicides on nitrification in a field soil under laboratory conditions. Weeds 5: 331-341.

Hays, J.T. 1967. Factors Affecting Performance of Urea-formaldehyde Fertilizers. Bull. Hercules Research Centre, Wilmington, Delaware.

_____, and W.W. Haden. 1966. Soluble fractions of urea-forms-nitrification, leaching and burning properties. J. Agri. Fd. Chem. 14: 339-341.

Hewitt, E.J. and D.J.D. Nicholas. 1963. Cations and Anions : Inhibitors and Interactions in Metabolism and in Enzyme Activity. In Metabolic Inhibitors, Vol. II., R.M. Hochster and J.H. Quastel (ed.) pp. 311-421. Academic Press, New York.

Hirose, S. and Y. Goto. 1961. Mode of adsorption of urea by rice seedlings. Abstr. Soil Sci. and Plant Nutr. 7: 85.

Hoagland, D.R. and T.C. Broyer. 1936. General nature of the process of salt accumulation by roots with description of experimental methods. Plant Physiol. 11: 474-507. (Quoted by Viets, 1965).

_____., _____. 1940. Hydrogen ion effects and the accumulation of salt by barley roots as influenced by metabolism. Am. J. Bot. 27: 173-185. (Quoted in Viets, 1965).

Hoflich, G. 1968. The effect of various chemical substances on nitrification, some soil microorganisms and leaching of nitrogen. Abstr. Soils and Fert. 32: 378.

Hoffman, T. and H. Lees. 1953. The biochemistry of the nitrifying organisms. 4. The respiration and inter-mediary metabolism of Nitrosomonas. Biochem. J. 54: 579-583.

Hoffman-Ostenhof, O. 1963. Enzyme Inhibition by Quinones. In Metabolic Inhibitors, Vol. II, R.M. Hochster and J.H. Quastel, (ed.) pp. 145-157. Academic Press, New York.

Jacques, R.P., J.B. Robinson, and F.E. Chase. 1959. Effects of thiourea, ethyl urethane and some dithio-carbamate fungicides on nitrification in Fox sandy loam. Can. J. Soil Sci. 39: 235-243.

Janert, R. 1968. Effect of N-serve on nitrification, plant growth and phosphorus uptake. Abstr. Soils and Fert. 31: 3327.

Jensen, H.L. 1951. Nitrification of oxime compounds by heterotrophic bacteria. J. Gen. Microbiol. 5: 360-368.

_____. and E. Bendixen. 1958. Ombiologisk virkning og sondirdiling of thiourinstof i jord og staldgodning. Tidsskr. Planteavl. 63: 499-520. (Quoted in McBeath, 1962).

_____. and H. Sorensen. 1952. The influence of some organic sulfur compounds and enzyme inhibitors on Nitrosomonas europaea. Acta Agr. Scand. 2: 295-304.

Jones, E.J. 1951. Loss of elemental nitrogen from soils under anaerobic conditions. Soil Sci. 71: 193-196.

Jones, G.A. 1968. Influence of autohydroxamic acid on some activities in vitro of the rumen microbiota. Can. J. Microbiol. 14: 409-416.

Kaempffe, G.C. and O.R. Lunt. 1967. Availability of various fractions of urea-formaldehyde. J. Agr. Fd. Chem. 15: 967-971.

Kluyver, A.J. and H.J.L. Donker. 1926. Die Einheit in der Biochemie. Chem. Zelle u. Geweke 13: 134-190. (Quoted in Alexander, 1965).

Kolenbrander, G.J. 1961. Leaching of nitrogen. De Fruittult, 51: 602-603.

Lees, H. 1946. Effect of copper-enzyme poisons on soil nitrification. Nature 158: 97.

_____. 1948. The effects of zinc and copper on soil nitrification. Biochem. J. 42: 534-538.

_____. 1963. Inhibitors of Nitrification. In Metabolic Inhibitors, Vol. II, R.M. Hochster and J.H. Quastel, (ed.), pp. 615-628. Academic Press, New York.

_____. and J.H. Quastel. 1945. Bacteriostatic effects of potassium chlorate on soil nitrification. Nature 155: 276-278.

_____. _____. 1946. Kinetics of and the effects of poisons on soil nitrification as studied by the soil perfusion technique. Biochem. J. 40: 803-814.

_____. and J.R. Simpson. 1955. The effect of chlorate on the rate of nitrate oxidation by Nitrobacter. Proc. Biochem. Soc. Biochem. J. 59: i.

_____. _____. 1959. The biochemistry of the nitrifying organisms 5. Nitrate oxidation by Nitrobacter. Biochem. J. 65: 297-305.

Lorenz, O.A. and C.M. Johnson. 1953. Nitrogen fertilization as related to the availability of phosphorus in certain California soils. Soil Sci. 75: 119-129.

Lunt, O.R. 1967. Sulfur-coated urea, a slow release fertilizer. Sulfur Inst. J., No. 2: 5-8.

_____. and J.J. Oertli. 1962. Controlled release of fertilizer minerals by encapsulating membranes. Part II. Efficiency of recovery, influence of soil moisture, mode of application, and other considerations related to use. Soil Sci. Am. Proc. 26: 584-587.

Mahendrappa, M.K. 1969. Determinations of nitrate-nitrogen in soil extracts using the specific ion activity electrode. Soil Sci. 108: 132-136.

Malingerova, N. 1964. The utility of polarographic nitrate determination in estimating the nitrifying power of soils. Abstr. Soils and Fert. 28: 818.

Marshall, K.C. and M. Alexander. 1962. Nitrification by Aspergillus flarus. J. Bacteriol. 83: 572-578.

Meiklejohn, J. 1954. Some aspects of the physiology of the nitrifying bacteria. In Autotrophic Microorganisms B.A. Fry and J.L. Pul, (ed.). 4th Symp. Soc. Gen. Microbiol. Cambridge Univ. Press. Cambridge, England.

Meyerhof, O. 1916. Untersuchungen uber den Atmungsvorgang
        nitrifizierender Bakterien.I. Die atmung des
        Nitrabildners. Pflug.Arch. ges. Physiol. 164:
        353-427. II. Buinflussung der atmung des nitrat-
        bildner's durch chemische Sustanzen. Pflug.
        Arch. ges. Physiol. 165: 229-284. III. Die Atmung
        des Nitribildners und ihre buinflussung durch
        chemische Substanzen. Pflug Arch. ges. Physiol.
        166: 240-280 (Quoted in Quastel and Scholefield,
        1951).

Murray, R.G.E. and S.A. Watson. 1965. Structure of Nitro-
        cystis oceanus and comparison with Nitrosomonas
        and Nitrobacter. J. Bact. 89: 1594-1609.

Myers, R.J.K. and E.A. Paul. 1968. Nitrate ion electrode
        method for soil nitrate-nitrogen determinations.
        Can. J. Soil Sci. 48: 369-371.

Nason, A. 1962. Enzymatic pathways of nitrate, nitrite
        and hydroxylamine metabolisms. Bact. Rev. 26: 16-41.

Nelson, C.E. 1953. Hydrocyanic acid content as affected
        by nitrogen fertilizer and soil moisture stress.
        Agron. J. 45: 615-618.

_____. 1967. Ammonium versus nitrate fertilization of
        field corn and related studies. Bull. Wash. Agr.
        Exp. Stn. No. 685: 11.

Nelson, J.L. 1957. Rapid determination of Nitrate and
        Nitrite. Analytical Chem. 26: 1081.

Nielson, K.F. 1967. Effect of chemical inhibition of
        nitrification on phosphorus absorption by wheat.
        Can. J. Soil Sci. 47: 65-71.

Nicholas, D.S.D. and O.T.G. Jones. 1960. Oxidation of
        hydroxylamine in cell-free extracts of Nitrosomonas
        europaea. Nature 185: 512-514.

Nightingale, G.T. 1948. II. Nitrogen nutrition of green
        plants. Botanical Rev. 14: 185-221.

Nishihara, T. 1962. The search for chemical agents which
        efficiently inhibit nitrification in soil, and studies
        on its utilization in agricultural practise. Abstr.
        Soils. and Fert. 26: 3072.

Nommik, H. 1967. Mineralization and availability of nitrogen in urea-formaldehyde compounds. Acta. Agr. Scand. 17: 33-38.

Nowakowski, T.Z. 1967. The effect of a nitrification inhibitor on the concentration of nitrates in plants. J. Agr. Sci. 68: 131-133.

_____. 1968. The effect of a nitrification inhibitor on the concentration of nitrate in grass during growth. Abstr. Soil and Fert. 31: 2552.

Oertli, J.J. and O.R. Lunt. 1962. Controlled release of fertilizer minerals by encapsulating membranes. Part I. Factors influencing rate of release. Soil Sci. Am. Proc. 26: 579-583.

Owens, L.D. 1960. Nitrogen movement of transformations in soils as evaluated by a lysimiter study utilizing isotopic nitrogen. Soil Sci. Am. Proc. 24: 372-376.

Parr, J.F. 1964. Possible approaches to the development of new nitrogen fertilizers. In Soil and Fertilizer Research Symposium Proceedings pp. 36-57. The Tennessee Valley Authority, Wilson Dam, Alabama.

_____. 1967. Biochemical considerations for increasing the efficiency of nitrogen fertilizers. Soils and Fert. 30: 207-213.

Pasteur, M.L. 1862. Etudes sur les myxodermes. Role de ces plantes dans la fermentation acetique. J. Exp. Bot. 7: 381-386. (Quoted in McBeath, 1962).

Patrick, W.H. Jr., F.J. Peterson, and F.T. Turner. 1968. Nitrification Inhibitors for Lowland Rice. Soil Sci. 105: 103-105.

Powell, R. 1968. Controlled release fertilizers. Chem. Proc. Rev. No. 15: 102-263.

Prasad, R. 1966. N-serve, 2-chloro-6-(trichloromethyl) pyridine for reducing losses from upland paddy soils. Abstr. Soils and Fert. 30: 426.

Quastel, J.H. and P.G. Scholefield. 1949. Influence of organic nitrogen compounds on nitrification in soil. Nature 164: 1068-1072.

_____. _____. 1951. Biochemistry of nitrification in soils. Bact. Rev. 15: 1-53.

Ray, H.E., J.M. McGregor and E.L. Schmidt. 1957. Movement of ammonium nitrate in soils. Soil Sci. Am. Proc. 21: 309-312.

Reddy, G.R. 1964. Effects of varying quantities of dicyandiamide on the utilization of nitrogen by several crops from $NaNO_3$ and $(NH_4)_2SO_4$. J. Agr. Sci. 62: 35-38.

Redemann, C.T., R.W. Meikle and J.G. Widofsky. 1964. The loss of 2-chloro-6-(trichloromethyl) pyridine from soil. J. Agr. Fd. Chem. 12:207-209.

Rehm, G.W. 1969. Effect of ammonium and nitrate nitrogen in sulfate uptake by corn. Univ. of Manitoba Ph.D. thesis. Dept. of Soil Sci.

Rich, S. and J.G. Horsfall. 1963. Fungicides as metabolic inhibitors. In Metabolic Inhibitors, Vol. II, R.M. Hochster and J.H. Quastel, (ed.) pp. 263-282. Academic Press, New York.

Richardson, H.L. 1938. Reduction of nitrate to ammonium for quantitative determinations of the former. J. Agr. Sci. 28: 73.

Rindt, D.W., G.M. Blouin, and J.G. Getsinger. 1968. Sulfur-coating on nitrogen fertilizer to reduce dissolution rate. J. Agr. Fd. Chem. 16: 773-778.

Robinson, J.B.D., M. de V. Allen, and P. Gacoka. 1959. Nitrate determinations via brucine method. Analyst 84: 635.

Sabey, B.R. 1968. The influence of nitrification suppressants on the rate of ammonium oxidation in mid-western U.S.A. field soils. Soil Sci. Am. Proc. 32: 675-679.

Sanderson, G.W., and E.C. Cocking. 1964. Enzymic assimilation of nitrate in tomato plants. I. Reduction of nitrate to nitrite. II. Reduction of nitrite to ammonium. Plant Physiol. 39: 416-431.

Schloesing. T. and A. Muntz 1877. Sur la nitrification par des ferments organises. Compt. Rend. Acad. Sci. (Paris) 85: 1018-1020. (Quoted by McBeath, 1962).

Shaw, N.M. 1960. Pesticide effects in soils on nitrification and plant growth. Soil Sci. 90: 320-323.

Skyring, G.W., B.G. Gary and V.B.D. Skerman. 1958. Polarographic method of nitrate determination. Soil Sci. 91: 388.

Sweezy, A.W. and G.O. 1962. Crop experiments on the effect of 2-chloro-6-(trichloromethyl) pyridine for the control of nitrification of ammonia and urea fertilizers. Agron. J. 54: 532-535.

Tandon, S.D. 1968. Effect of some rare elements on nitrification by nitrate-forming bacteria in talc medium. Agrochimica 12: 365-368.

Thompson, F.R. 1969. Persistence and effects of some chlorinated anilines on nitrification in soil. Can. J. Micro. 15: 791-796.

Tomlinson, T.G., A.G. Boon and G.N.A. Trotman. 1966. Inhibition of Nitrification in the Activated Sludge Process of Sewage Disposal. J. Applied Microbiol. 29: 266-291.

Toogood, J.A. and T.W. Peters. 1953. Comparison of mechanical analysis of soils. Can. J. Agr. Sci. 33: 159-171.

Turner, G.O., L.E. Warren and F.G. Andriessen. 1962. Effect of 2-chloro-6-(trichloromethyl)pyridine on nitrification of ammonium fertilizers in field soils. Soil Sci. 94: 270-273.

Tyler, K.B. and F.E. Broadbent. 1960. Nitrite transformation in California soils. Soil Sci. Am. Proc. 24: 279-282.

Wagner, G.H. and G.E. Smith. 1958. Nitrogen losses from soils fertilized with different nitrogen carriers. Soil Sci. 85: 125-129.

Warington, R. 1878. On nitrification.I. J. Chem. Soc.
        33: 44-51.

_____. 1879. On nitrification. II. J. Chem. Soc.
        35: 429-456.

_____. 1884. On nitrification. J. Chem. Soc. 45: 637.

_____. 1891. On nitrification. J. Chem. Soc. 59: 484.

Wetselaar, R. 1962. Nitrate distribution. III. Downward
        movement and accumulation of nitrate in the subsoil.
        Plant and Soil. 16: 19-31.

Wilson, H.A. 1954. The Effect of Certain Pesticides on
        Nitrification in Soil. W. Va. Agr. Exp. Sta.
        Bull. 3667.

Winely, C.L. 1969. The effect of pesticides on nitrite
        oxidation by Nitrobacter agilis. Bact. Proc. 1969.
        A22.

Winogradsky, S. 1890. Recherches sur les organismes de la
        nitrification. Ann. Inst. Pasteur. 4: 213-231,
        257-275, 760-771. (Quoted in McBeath, 1962).

_____. 1891. Recherches sur les organismes de la nitrifi-
        cation. Ann. Inst. Pasteur 5: 32-100, 577-616.
        (Quoted in McBeath, 1962).

_____. and V. Dmeliansky. 1899. L'influence des substances
        organiques sur le travail des microbes nitrifica-
        teures. Arch. Sci. Biol. 7: 1-39 (Quoted in McBeath,
        1962).

Viets, F.G. Jr. 1965. The Plants' need for and use of Nitro-
        gen. In Soil Nitrogen, W.V. Bartholomew and F.E.
        Calrk (ed.) A.S.A. Monograph 10. Am. Soc. Agron.
        Madison, Wisconsin.

_____, L.C. Boawn and C.L. Crawford. 1957. The effect of
        nitrogen and type of nitrogen carrier on plant uptake
        of indigenous and applied zinc. Soil Sci. Am. Proc.
        21: 197-201.

Zavarzin, G.A. 1958. The incitation of the second stage of
        nitrification. II. The effect of heavy metals on
        nitrification. Mikrobiologiya 27: 542-546.

APPENDIX I

CHEMICAL COMPOUNDS EVALUATED IN THIS STUDY FOR

THE ABILITY TO INHIBIT NITRIFICATION IN SOIL

Organo-Nitrogen Compounds

Bis(salicylaldehyde) ethylenediimine
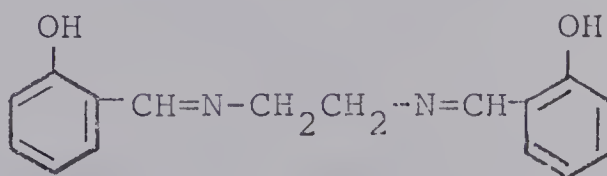
2-nitroso-1-mapthol
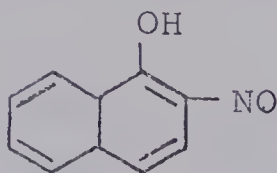
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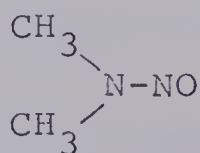
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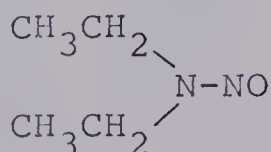
1,2 Diamino-NN'-bis(methyl-2-hydroxy-5-methylbenzyl) ethane
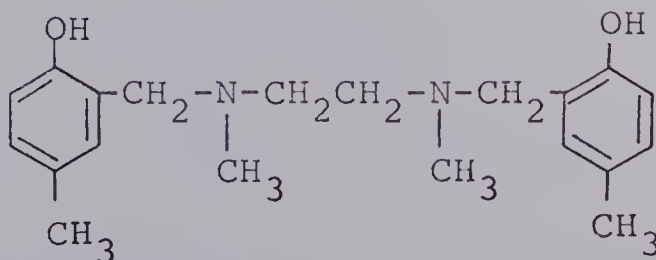
2-pyridinehldoxime
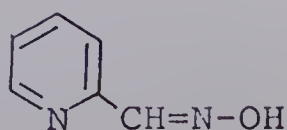
Hexamethylenetetramine
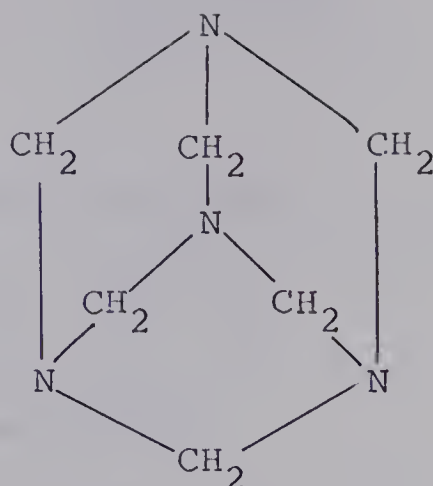
1,2-diamino-N,N^1-bis(methyl-1-hydroxy-5-methylbenzyl) ethane
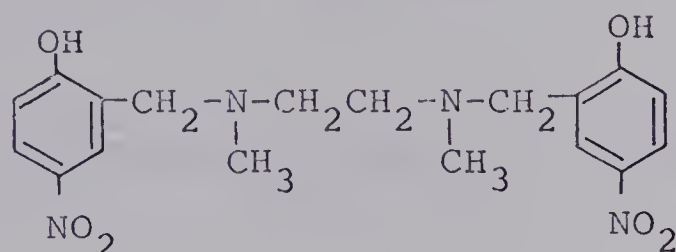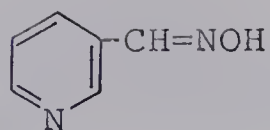

Diacetylmonoxime-p-nitrophenylhydrazone
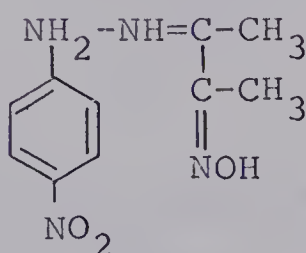
Benzoin oxime
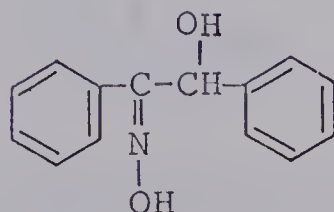
N,N(dimethyl)-N-(2-hydroxy-3-methylbenzyl) amine

3-Pyridinealdoxime

$$\text{pyridine ring} - CH=NOH$$

α-Nitroso-β-chloromethylacrylate

$$\underset{\underset{Cl}{|}}{CH_2} - \underset{\underset{NO}{|}}{CHC} \overset{O}{\overset{||}{C}} - OCH_3$$
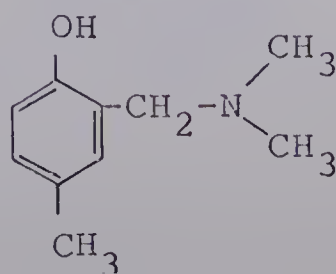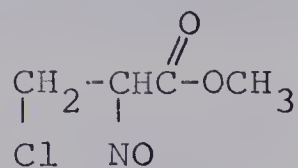
NOCl adduct of trans-stilbene

$$Ph - \underset{\underset{}{|}}{CH} - \underset{\underset{Cl}{|}}{CH} - Ph \quad (NO \text{ on first } CH)$$
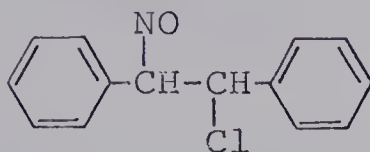
HCl salt of a chlorination product of 2-pyridinealdoxime

$$\underset{\underset{H}{\overset{+}{N}}}{\text{pyridinium}} - \underset{\underset{Cl}{|}}{C} = NOH \qquad Cl^-$$

Chlorination product of 2-pyridinealdoxime

$$\underset{\underset{N}{}}{\overset{Cl}{\text{pyridine}}} - \underset{\underset{Cl}{|}}{C} = NOH$$

Chlorination product of 2-pyridinealdoxime

$$\underset{N}{\text{pyridine}} - \underset{\underset{Cl}{|}}{C} = NOH$$
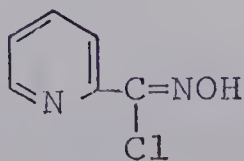
Pyridine-2,6-dicarboxylate
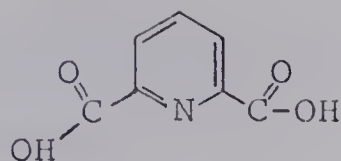
Organo-Nitrogen-Sulfur Compounds

Thiocyanuric acid
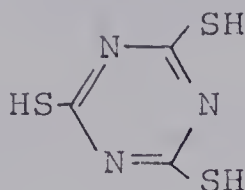
3-Thiosemicarbazide

$$NH_2-\overset{\overset{\displaystyle S}{\|}}{C}-NHNH_2$$
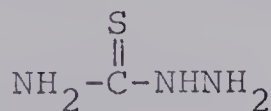
α-Mercaptopyridine
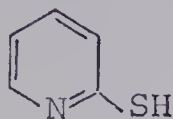
Thiouric acid
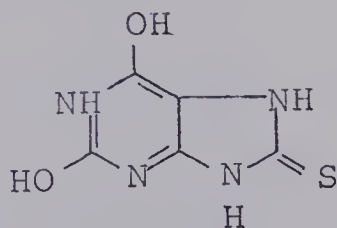
N(1-methoxybutyl) thiourea

$$NH_2-\overset{\overset{\displaystyle S}{\|}}{C}-NH-\overset{\overset{\displaystyle OCH_3}{|}}{CH}-CH_2CH_2CH_3$$

N(1-hydroxybutyl) thiourea

$$\underset{\substack{\| \\ NH_2-C-NH-CH-CH_2CH_2CH_3}}{\overset{\substack{S \quad\quad OH \\ \| \quad\quad |}}{}}$$

N(1-hydroxypropyl) thiourea

$$\underset{\substack{| \quad\quad | \\ S \quad\quad OH}}{NH_2-C-NH-CHCH_2CH_3}$$

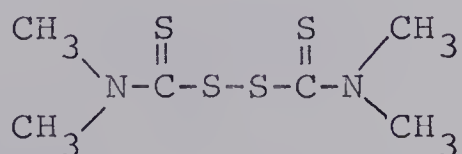Dithiooxamide

$$\underset{\substack{\| \;\; \| \\ S \;\; S}}{H_2N-C-C-NH_2}$$
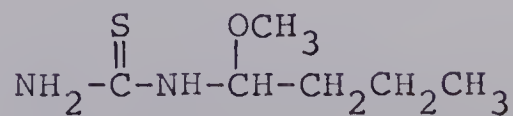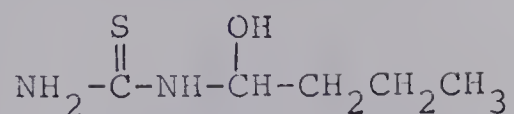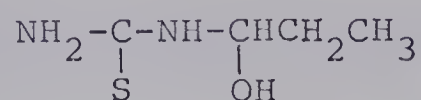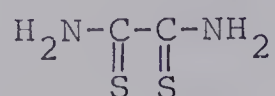
1,2-Diamino-N,N'(methyl-2-mercapto-5-methylbenzyl) ethane
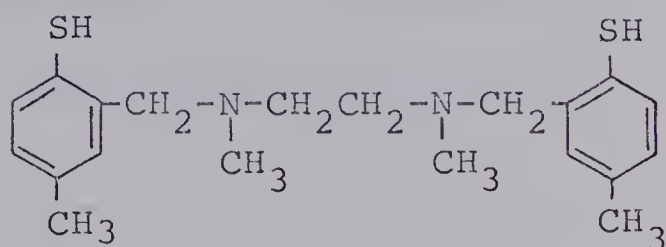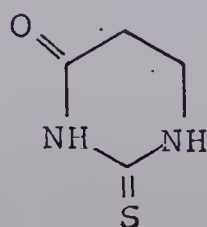

Bis(dimethylthiocarbamyldisulfide)

$$\underset{\substack{CH_3 \diagdown \\ \\ CH_3 \diagup}}{}\underset{\substack{S \quad\quad S \\ \| \quad\quad \| \\ N-C-S-S-C-N}}{}\underset{\substack{\diagup CH_3 \\ \\ \diagdown CH_3}}{}$$

2-Mercaptobenzothiazole

β-Phenylthiosemicarbazide

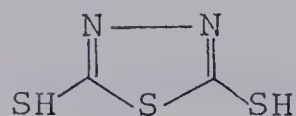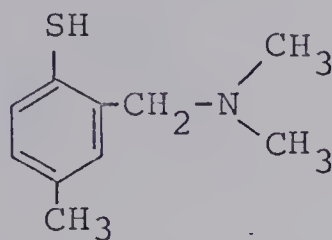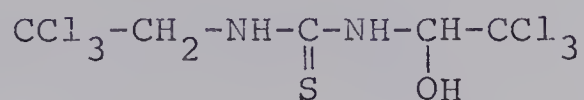$$\underset{\substack{\| \\ NH_2-C-NHNH-}}{\overset{S}{}}\bigcirc$$
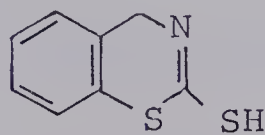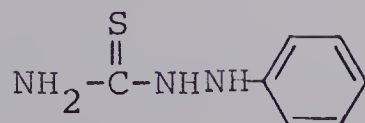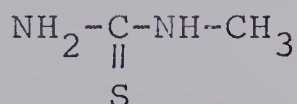
2-Thiouracil

2,5-Dimercapto-1,3,4-thiadiazole
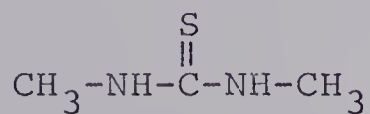

N,N(dimethyl)N(2-mercapto-5-methylbenzyl)amine

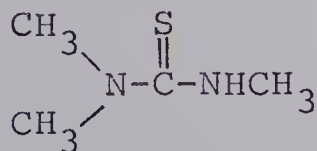N(2,2,2-trichloroethyl)-N'-(1-hydroxy-2,2,2-trichloroethyl) thiourea

$$CCl_3-CH_2-NH-\underset{\underset{S}{\|}}{C}-NH-\underset{\underset{OH}{|}}{CH}-CCl_3$$

N-(methylthiourea)

$$NH_2-\underset{\underset{S}{\|}}{C}-NH-CH_3$$

N,N'-(dimethyl)thiourea

$$CH_3-NH-\overset{\overset{S}{\|}}{C}-NH-CH_3$$

N,N(dimethyl)-N'-(methyl)thiourea

$$\underset{CH_3}{\overset{CH_3}{>}}N-\overset{\overset{S}{\|}}{C}-NHCH_3$$

N-acetylthiourea

$$CH_3\overset{\overset{O}{\|}}{C}-NH-\overset{\overset{S}{\|}}{C}-NH_2$$

1-Allyl-2-thiourea

$$NH_2-\underset{\underset{S}{\|}}{C}-NH-CH_2CH{=}CH_2$$

Foramidine sulfinic acid

$$HC-\underset{\underset{O}{\|}}{C}-\underset{\underset{NH}{\|}}{N}H-S\overset{\overset{O}{\|\!\!\!\!\diagup}}{\diagdown}_{OH}$$

2-Allyl-2-thiopsuedourea

$$CH_2{=}CH-\underset{\underset{\underset{\underset{HN{=}C-NH_3\bar{C}l}{+}}{S}}{|}}{CH}_2$$
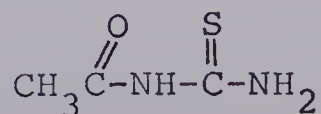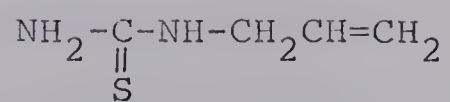
Polymers

55-67	Urea-thiourea copolymer
66-67	Phenol-thiourea copolymer
67-67	Thiophenol-thiourea
68-67	Butryraldehyde-formaldehyde-thiourea polymer
MW41	Methylene-urea-thiourea polymer

$$(NH_2-\underset{\underset{S}{\|}}{C}-NH-CH_2-NH-\underset{\underset{O}{\|}}{C}-NH-CH_2-NH-\underset{\underset{S}{\|}}{C}-NH_2)$$

P34	N,N^1(dimethyl) thiourea dimer

$$(CH_3-NH-\underset{\underset{S}{\|}}{C}-NH-CH_2-CH_2-NH-\underset{\underset{S}{\|}}{C}-NH-CH_3)$$

305-15	Thiourea-formaldehyde polymer (low molecular weight)
305-28	Thiourea-formaldehyde polymer (high molecular weight)
118-66-IC	Thiourea-formaldehyde polymer (low molecular weight)
118-66-16	Thiourea-formaldehyde polymer
305-25-B	Thiourea-formaldehyde polymer
305-14	Thiourea-formaldehyde polymer
307-18	thiourea-formaldehyde polymer
307-25	Thiourea-formaldehyde polymer
307-26A	Thiourea-formaldehyde polymer
27-1	Thiourea-formaldehyde polymer
27-2	Thiourea-formaldehyde polymer
27-3	Thiourea-formaldehyde polymer
306-5	Thiourea-formaldehyde polymer

305-51	Thiourea-formaldehyde polymer
307-36	Thiourea-formaldehyde polymer
307-36	Thiourea-formaldehyde polymer
307-39B	Thiourea-formaldehyde polymer
307-36A	Thiourea-formaldehyde polymer
MW 30-1	Thiourea-formaldehyde polymer
MW 30-2	Thiourea-formaldehyde polymer
308-4	Thiourea-formaldehyde polymer
305-28	Thiourea-formaldehyde polymer
308-2	Thiourea-formaldehyde polymer
308-7	Thiourea-formaldehyde polymer

Appendix II

Chemical and Physical Properties of Malmo Clay Loam

[1]pH	6.4
[2]Cation Exchange Capacity	37 mg/100 gm
[3]Carbon Content	6.4%

[4]Mechanical Analysis

% sand	29
% silt	39
% clay	32

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[1]Doughty, 1941.

[2]Association of Official Agricultural Chemists, 1955.

[3]Allison, Bollen and Moodie, 1965.

[4]Toogood and Peters, 1953.

Appendix III
Indicator Reagents for Qualitative
Nitrate and Nitrite Ion Detection

Trommsdorf's Reagent for Nitrites

1. Add slowly with constant stirring, a boiling solution of 20 gm. of zinc chloride in 100 mls. of distilled water to a mixture of 4 gm. of starch in water. Continue heating until the starch is dissolved as much as possible and the solution is clear.

2. Dilute with water and add 2 gm. of zinc iodide; dilute to 1 litre with water and filter.

3. Store in well-stoppered bottle and keep it in a dark place.

4. A 1:3 solution of dilute sulphuric acid is the complementary test solution. This is stored separately.

Diphenylamine Reagent for Nitrites and Nitrates

1. Dissolve 0.7 gm. of diphenylamine in a mixture of 60 ml. of concentrated sulphuric acid and 28.8 ml. of distilled water.

2. Cool this mixture and add slowly 11.3 ml. of concentrated HCl (Specific gravity 1.19). Let stand overnight.

3. After standing overnight, some of the bases will separate, showing that the reagent is saturated.
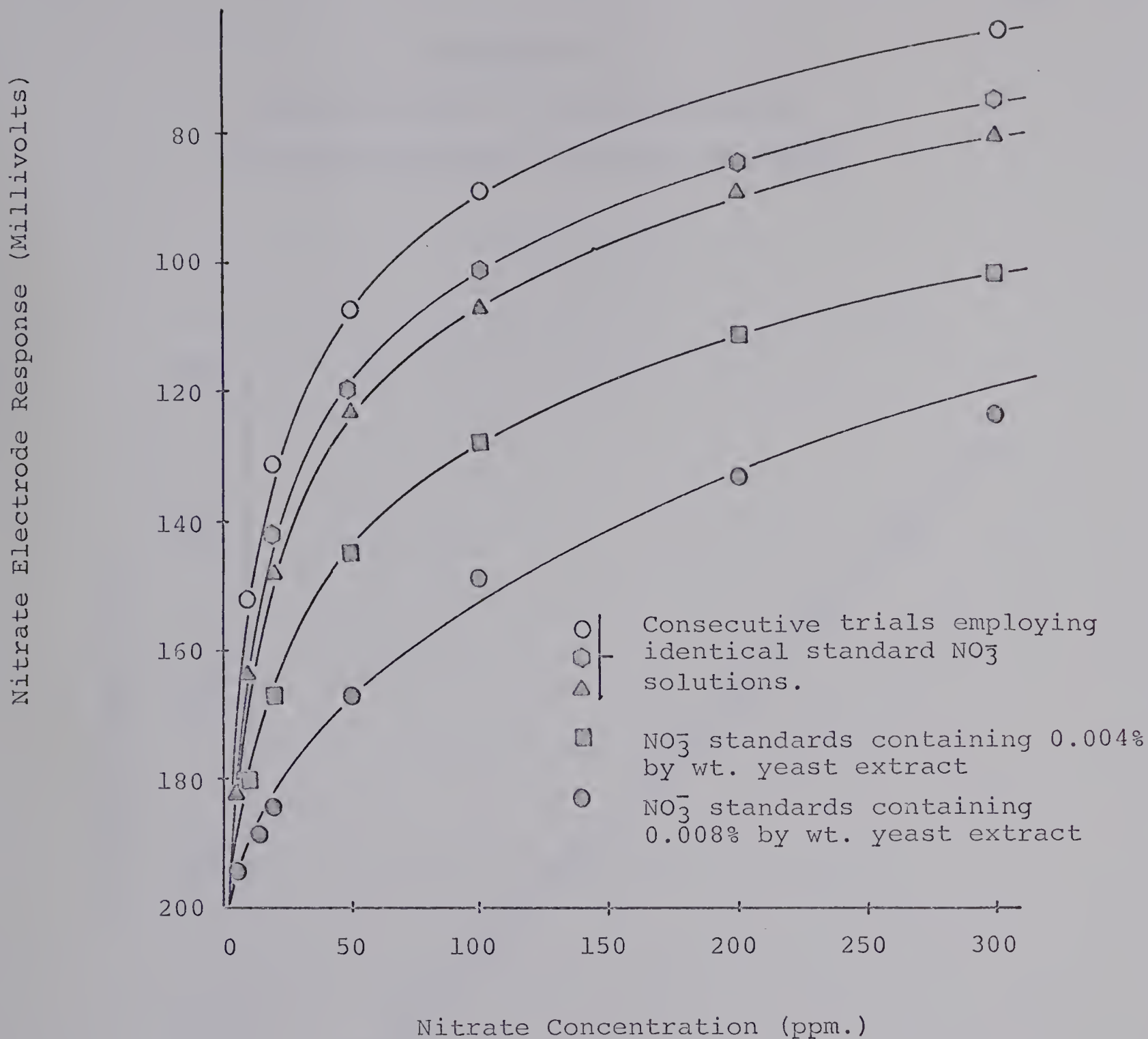
Experimental calibration curves for the nitrate ion electrode, Model 92-07 (Orion Research Inc., Cambridge, Mass., U.S.A.); illustrating its non-reproduceable behavior and sensitivity to interference by organic matter.

Electrode response monitored by Beckman (Model 76), expanded scale pH meter. Solutions maintained at 20°C and stirred magnetically. Time to electrode response stabilization - 3 minutes.

Appendix V

Standard Curve. Phenoldisulphonic

Acid Determination of Nitrate-Nitrogen

Klett-Summerson Colorimeter Reading

(420-Blue Filter)
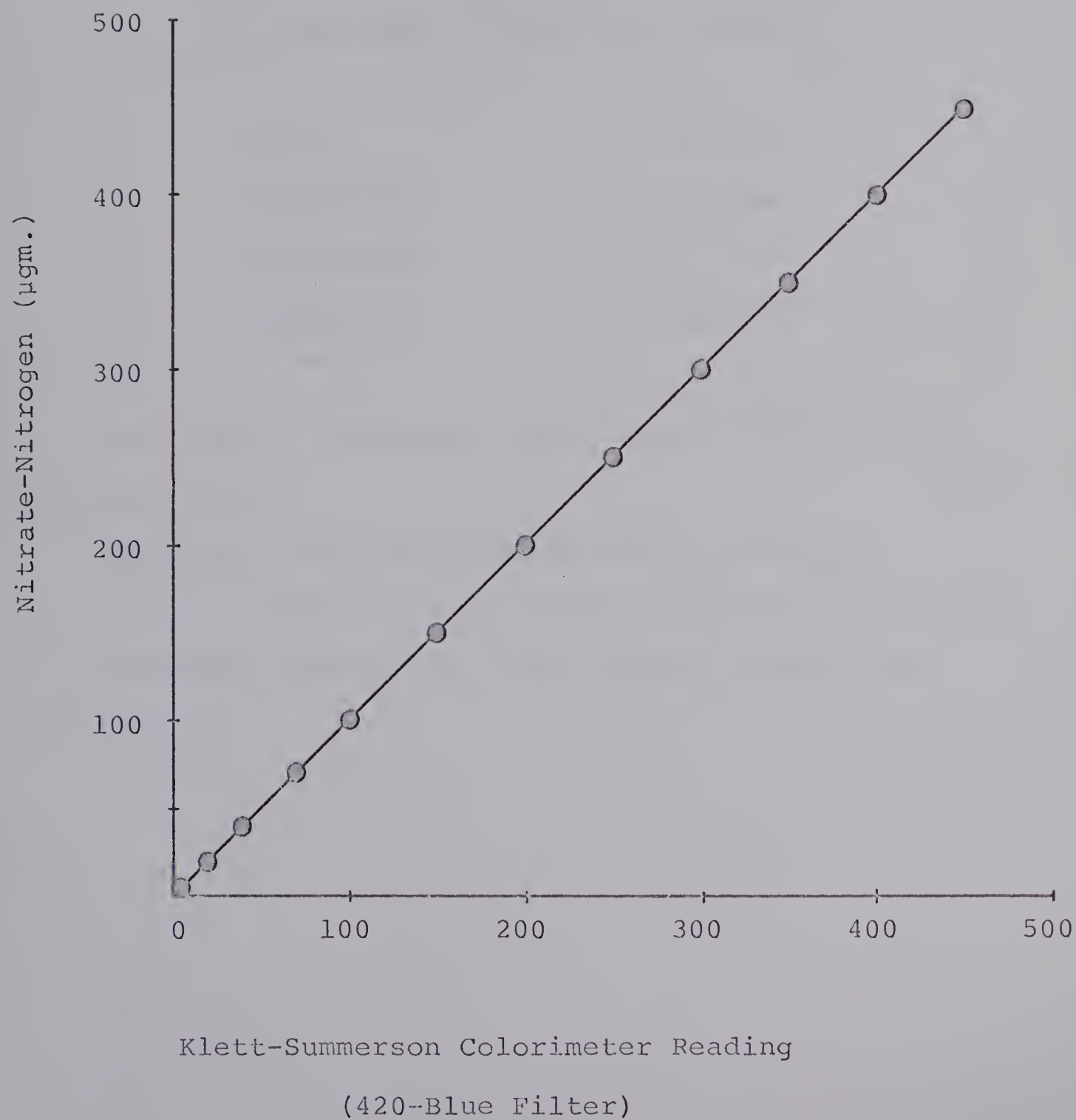
Appendix VI

Composition of Growth Medium for the Enrichment

of Autotrophic Nitrifying Bacteria

NaCl	0.3 gm.
$MgSO_4 \cdot 7H_2O$	0.14 gm.
$FeSO_4 \cdot 7H_2O$	0.03 gm.
$(NH_4)_4SO_4$	0.66 gm.

Dissolve the chemicals listed above in 90 ml. of distilled water.

Add 10 ml. of previously boiled 0.1 M kH_2PO_4.

For use, dilute 10 mls of this stock solution to 100 ml. with water, add excess (2 gm./100 ml.) $CaCO_3$ and sterilise.

Appendix VII

Specifications of Four Inhibitor-Coated

Urea-Products

Product	Test Inhibitor Employed as Coating	% Content in Final Product		
		Urea	Inhibitor	Kaolin
5-68	[1]118-66-IC	88.5	4.4	7.1
6-68	[2]305-14	86.2	4.3	9.5
7-68	[3]305-25B	87.0	4.3	8.7
8-68	2-Pyridinealdoxime	84.5	4.2	11.3

[1,2,3]Low molecular weight thiourea-formaldehyde polymer